Effects of Hydrogen Peroxide on Lipoproteins and Associated Lipids[1]

DALE A. CLARK, EMMETT L. FOULDS, JR. and FREDRICK H. WILSON, JR.,
Box 4243, USAF School of Aerospace Medicine, Brooks Air Force Base, Texas 78235

ABSTRACT

Lipoproteins isolated from human or chimpanzee serum were treated with H_2O_2 and allowed to stand varying lengths of time before quantitative analysis in the ultracentrifuge. Marked instability of ultracentrifugal boundaries (convection) occurred during the first 24 hr, but diminished thereafter. Simultaneously, the quantity of lipoprotein decreased. The instability of boundaries in H_2O_2-treated samples was presumed to reflect loss of lipid-protein affinity and breakdown of lipoproteins under the force of ultracentrifugation. Analysis of extracted lipids showed that H_2O_2 caused little loss of phospholipid, significant loss of triglyceride, and apparent loss of cholesteryl ester. The latter loss, however, was accompanied by appearance of esterified cholesterol in the free cholesterol eluent. Apparently H_2O_2 converted some cholesteryl esters to a more polar form which was eluted later from the column, with the free cholesterol fraction. Gas chromatographic analysis of the fractions eluted from the column showed that selective degradation of polyunsaturated fatty acids was most marked with cholesteryl esters, somewhat less with triglycerides, and negligible with phospholipids. It was postulated that the loss of lipid-protein affinity caused by H_2O_2 in vitro may reflect a similar process in vivo, i.e., that one process contributing to development of atherosclerosis can be oxidation of lipoprotein, with loss of lipid-protein affinity and accumulation of lipid products in (or on) cells of the vascular system.

INTRODUCTION

IT IS WIDELY RECOGNIZED that in human populations elevated levels of serum lipid (1,2) and lipoproteins (3) are attended by an increased risk of atherosclerotic heart disease. From the vast amount of research directed toward elucidating the role of elevated lipid levels in the development of atherosclerosis, it has become apparent that multiple factors and complex relationships are involved (4). The offending net effect of the interaction of these factors is the accumulation of cholesterol and other lipids in plaques on the walls of blood vessels.

Since the cholesterol in plaques apparently originates primarily from serum cholesterol (5), the process(es) by which cholesterol is converted from its solubilized state as a component of serum liproproteins into its insoluble state as a component of atherosclerotic plaques is (are) of crucial importance. It is conceivable that a high level of cholesterol in the serum might be metabolically acceptable if the cholesterol stayed in the serum. However, the problem of atherosclerosis is associated with the fact that the cholesterol does not stay in the serum, but as a result of unclarified processes, perhaps in cells of the blood vessel walls, accumulates on the walls of the vascular system in plaques along with other lipid, fibrous and mineral components. The present study considers possible mechanisms by which lipids, especially cholesterol, may become separated from their serum lipoprotein complexes.

Several lines of evidence indicate that peroxidation may play a role in atherosclerosis. The presence of peroxidized lipids in atherosclerotic plaques was demonstrated by Glavind and Hartman (6), who found lipid peroxides in plaques, while these materials were absent from normal vascular tissue. Ceroid pigment, which is formed by peroxidation of unsaturated lipids, is found in atherosclerotic plaques and was reported by Hartroft to be the only lipid of such plaques not common to the serum lipids (7). An association between peroxidation and atherosclerosis was also indicated by the work of Caravaca et al. (8), who found that intramuscular injections of beef liver peroxidase into rabbits being fed a cholesterol-enriched diet resulted in development of markedly less atherosclerosis than in nonperoxidase-injected controls. It is well established that serum lipoproteins are highly subject to oxidation (9). In fact, special precautions are required during isolation of serum lipoproteins

[1]Presented at the AOCS Meeting, Washington, D. C., April, 1968.

TABLE I

Effects of Hydrogen Peroxide on Quantities of Lipoproteins Subsequently Measured Ultracentrifugally

Exposure time[a]	Sf 0–12			Sf 12–20			Sf 20–400			High Density		
	Control level mg/100cc	n	100 P[b] ± SD / C	Control level mg/100cc	n	100 P ± SD / C	Control level mg/100cc	n	100 P ± SD / C	Control level mg/100cc	n	100 P ± SD / C
½ hr	189-224	3	106 ± 9	6-28	3	125 ± 48	40-100	3	109 ± 28	172-318	3	20 ± 18
1 hr	173-352	6[c]	94 ± 12	4-58	6[c]	95 ± 54	8-183	6[c]	240 ± 140	123-215	3	82 ± 20
2 hr	156-240	2	62 ± 51	7-68	2	63 ± 75	58-206	2	114 ± 22	207-235	2	62 ± 48
24 hr	150-303	10[c]	41 ± 27	4-61	9[c]	19 ± 30	16-208	10[c]	164 ± 225	116-237	4	25 ± 22
28 hr	152-378	7[c]	36 ± 31	4-51	6[c]	4 ± 9	8-176	7[c]	136 ± 82			
72 hr	170-185	2	0	4	2	0	24-50	2	60 ± 85			

[a] Time from addition of H_2O_2 to insertion of cell in rotor. An additional 17 min pumpdown time and 6 min acceleration time elapsed before rotor was up to speed.

[b] P = lipoprotein concentration in peroxide-treated aliquot; C = lipoprotein concentration in control aliquot. The mean and standard deviation of the indicated (n) samples are shown. The SD includes variation due to analytical error as well as variation among samples in their susceptibility to degradation by peroxide.

[c] The n runs include 3 runs with pooled chimpanzee low density liproproteins. The remainder of the n runs and all n's not marked c were made with human lipoproteins.

or lipids to prevent autoxidation (10). The hypothesis was therefore entertained that peroxidation of lipoproteins might occur in vivo either in serum or in cells in walls of arteries and cause loss of lipid-protein affinity, resulting in loss of lipid-solubilizing capacity of the apoproteins, and accumulation of lipid in plaques.

To test one facet of this hypothesis, lipoproteins were separated from serum by ultracentrifugation. Aliquots of lipoproteins were then reacted with hydrogen peroxide, and analyzed for lipid and for lipoprotein content. Control aliquots of these same lipoprotein preparations were not reacted with peroxide, but were similarly analyzed for lipid and lipoprotein content.

EXPERIMENTAL PROCEDURES

Liproproteins were isolated from serum of human or chimpanzee subjects and measured quantitatively by the method of DeLalla and Gofman (11), except that KBr was used instead of D_2O to increase density (12) to 1.20 for separation of high density lipoproteins at 26 C. and 52,640 rpm. In one experiment three different pools of lipoproteins from chimpanzee sera were used (Table I). In all other experiments human serum lipoproteins were used. Sufficient serum was obtained from each subject to permit 2 to 4 replicate preparative runs. The top fractions from the replicate preparative runs from a given individual serum sample were combined to yield a pool of low density ($d \leq 1.063$) and another pool of high density ($1.063 \leq d \leq 1.20$) lipoproteins from each subject. Each such lipoprotein pool from individual serum sample was divided into at least two 1.0 ml aliquots, to one of which was added 50 λ of 12% aqueous H_2O_2 (final conc. = 186 mM). To another 1.0 ml aliquot (the control) was added 50 λ of water.

Aliquots to be used within 2 hr were left at room temperature (23-25 C.) until use. Aliquots to be used later were refrigerated at 8 C. immediately after H_2O_2 was added. One hour before use, they were withdrawn, warmed in H_2O at room temperature, and left standing until start of pumpdown for ultracentrifugation.

The effects of hydrogen peroxide in serum were also studied by adding 100 λ of 12% H_2O_2 to 1 ml serum (final conc. H_2O_2 = 355 mM). To another 1 ml of the same serum was added 100 λ H_2O for a control. Both aliquots were processed identically thereafter for lipoprotein preparation and quantitative analysis.

After incubation of either serum or lipoprotein fractions at room temperature for various periods of time, an aliquot of control and of peroxidized samples was taken for quantitative determination of lipoprotein levels. In all instances, care was taken to compensate for the volumes and densities of various solutions added and to adjust solution densities to 1.063 or 1.20 for preparation and quantitative measurement of low and high density lipoproteins, respectively. For instance, H_2O_2 (and H_2O) dilutions were prepared in D_2O in order to permit subsequent adjustment to the desired density.

Aliquots of control and peroxidized samples were also taken for extraction of lipids, separation by chromatography over silicic acid, and quantitative determinations by previously described methods (13). In some instances,

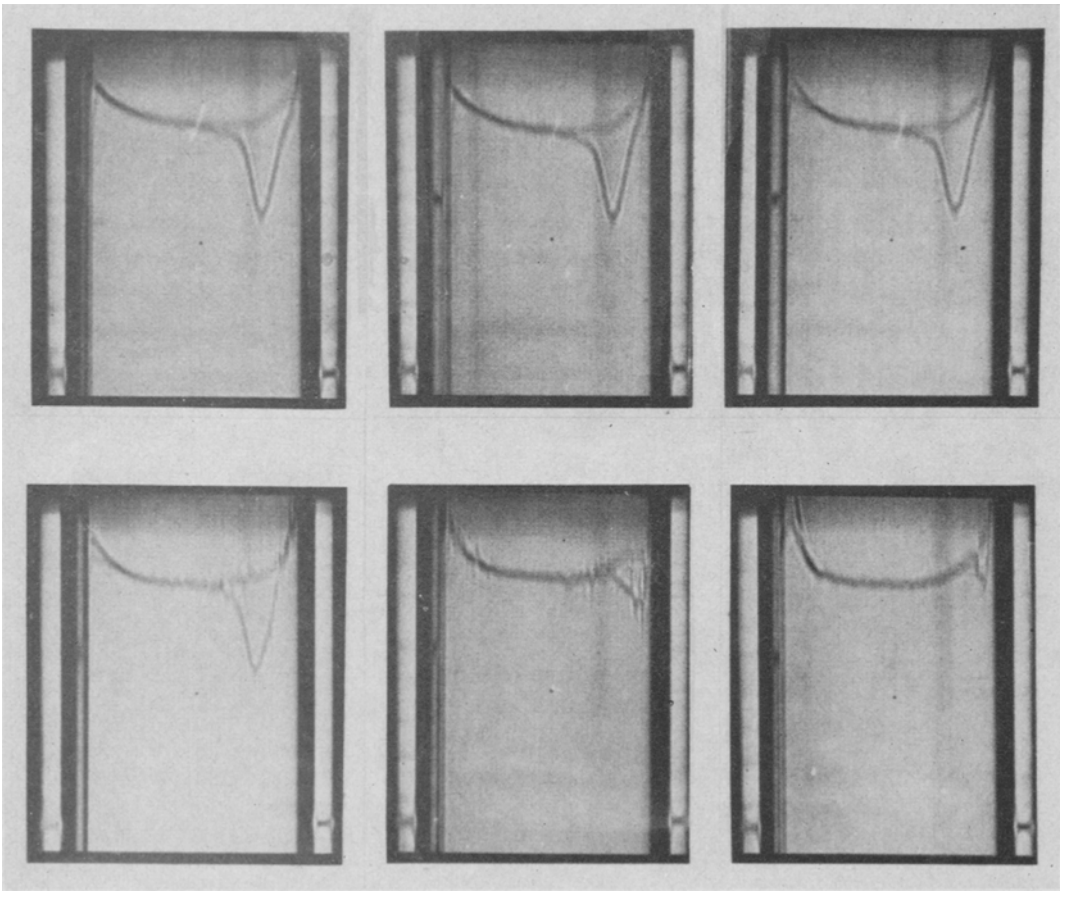

FIG. 1. Effect of H_2O_2 on low density liproproteins from pooled chimpanzee serum observed in fifth frame (33 min) of ultracentrifugal analytical runs. After addition of H_2O (top row) or H_2O_2 (bottom row), aliquots stood for time intervals of (left to right) 1 hr at 23 C, 24 hr (1 hr at 23 C+ 23 hr at 8 C), or 48 hr (1 hr at 23 C +47 hr at 8 C) before start of run.

aliquots of fractions from the columns were saponified and the methyl esters of fatty acids prepared and analyzed by gas chromatography. Phospholipid fatty acids were transmethylated by the method of Morrison and Smith (14). Methyl esters of the fatty acids in cholesteryl esters and triglycerides were saponified by the method of Metcalfe et al. (15), and methylated by the method of Morrison and Smith (14). Accuracy of gas chromatographic analyses was determined by linearity testing with NIH Metabolism Study Section Fatty Acid Mixtures A-F. Fatty acids were identified by comparison with relative retention times of known standards. A Loenco 70 Hi-Flex gas chromatograph with dual flame ionizaton detectors was used. Gas chromatography was effected with a 7 ft × ¼ in. O.D. column packed with 20% DEGS on 60–80 mesh Chromosorb W silanized with HMDS (Loenco, Inc., Altadena, Calif.) and operated at 198 C.

RESULTS

Isolated lipoproteins were degraded by hydrogen peroxide. The difference in schlieren patterns of control and peroxidized samples is presented in Fig. 1 and 2. All those patterns were photographed during regular lipoprotein runs. Marked convective disturbances were apparent in the schlieren patterns of peroxide-treated aliquots after 1 hr contact with H_2O_2 (Fig. 1 and 2). The severity of convective disturbance usually was maximal after 4–24 hr contact with H_2O_2, after which the severity of the disturbance decreased. After peroxide addition, a progressive decrease in lipoprotein content was observed. This de-

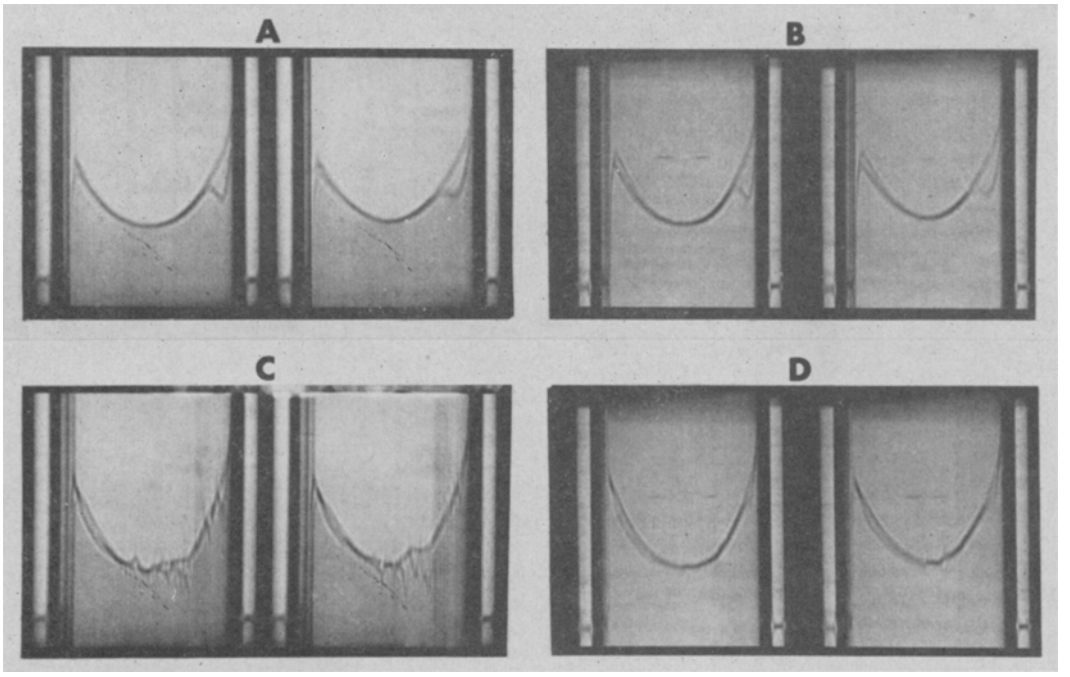

FIG. 2. Effect of H_2O_2 on high density lipoproteins from human serum observed 32 and 48 min (left and right, respectively, in each pair of photos) after start of ultracentrifugal analytical runs (11). After addition of H_2O (A and B) or H_2O_2 (C and D), aliquots stood for time intervals of 1 hr at 23 C (A and C) or 1 hr at 23 C plus 23 hr at 8 C (B and D) before start of run.

crease is shown in the S_f 0–12 fraction over a 48 hr period in Fig. 1 (cf. area between curves in bottom row vs. respective controls in top row). A marked decrease in the concentration of high density lipoproteins during a 24 hr period is shown in Fig. 2.

The severe convective disturbances evident in Fig. 1 and 2 were observed only in the peroxide-treated aliquots. Minor convective disturbances were observed in some controls. However, in samples where the lipoprotein had been markedly decreased by peroxide treatment, the convective disturbance was minor or even absent, whereas an aliquot of the same sample at an earlier time showed less loss of lipoprotein but greater boundary disturbance. The tentative explanation of this phenomenon, therefore, is that the convective disturbances are the result of breakdown of lipoprotein structure and actual separation of lipid and protein during the ultracentrifuge run, causing discontinuities in the boundaries of the migrating lipoproteins.

In the presence of the marked disturbances of convection, quantitative estimation of the various lipoproteins was grossly inaccurate. However, estimates were made using care to err on the side of overestimation, rather than underestimation of lipoprotein concentrations. The data recorded in Table I show that lipoproteins were affected by hydrogen peroxide in the concentration used. The time of contact of H_2O_2 with the lipoproteins was obviously important. The decrease in lipoproteins after 53 min contact with H_2O_2 at room temperature (plus 17 min pumpdown and 6 min rotor acceleration time) was minimal, with obvious effects apparent only in the high density fraction. After 2 hr standing at room temperature (plus pumpdown and acceleration time) the S_f 0–12 and S_f 12–20 fractions were also affected in some samples. After 24 hr (23 hr refrigeration at 8 C. + 1 hr at 23–25 C. plus pumpdown and acceleration time) only the S_f 20–400 fraction appeared unaffected, but some of the products from the S_f 0–12 or S_f 12–20 or both lipoproteins apparently were measured in the S_f 20–400 fraction. With further standing, progressive decrease of lipoprotein fractions occurred until at 72 hr even the S_f 20–400 fraction was affected in most samples. These general relationships indicate that the order of sensitivity to H_2O_2 is high density

TABLE II

Lipoprotein Levels After Addition of H_2O_2 to Serum, 10 Sample[a]

	S_f 0–12	S_f 12–20	S_f 20–400	High density
Control levels, mg/100cc	134 - 291	4 - 38	20 - 159	152 - 393
Mean $100 \times \frac{P}{C} \pm SD$	101 ± 5	96 ± 25	107 ± 23	95 ± 14

[a] H_2O_2 was added to a final concentration of 355 mM to serum and allowed to stand at room temperature 2 to 3 hr before used in a preparative lipoprotein run. Control aliquots receive equal volume of H_2O instead of H_2O_2.

lipoproteins > S_f 0–12 > S_f 12–20 > S_f 20–400.

There were obvious differences in the extent of lipoprotein decreases effected by H_2O_2 when lipoproteins isolated from different subjects were compared. Such differences doubtless account for some of the wide standard deviations in the means of the data of Table I. Since antioxidants or proteins in the serum might explain this different susceptibility to oxidation, the effects of H_2O_2 added to serum were studied When H_2O_2 was added to serum and allowed to stand at room temperature up to 3 hr before a preparative lipoprotein run, no change in lipoprotein levels was caused by H_2O_2 (Table II) in serum samples as a group. However, in a few serum samples the high density lipoproteins were decreased by H_2O_2. For example, in one instance high density lipoprotein levels in duplicate controls were 281 and 336 while levels in duplicate peroxide-treated aliquots were 229 and 192. The low density lipoprotein levels of control and peroxide-treated aliquots of this serum, however, were not significantly different.

To ascertain the chemical effects of H_2O_2 on lipoprotein lipids, aliquots of isolated lipoprotein solutions treated with H_2O_2, but not used for ultracentrifugal analysis, were extracted with chloroform-methanol (2:1) and the lipids fractionated by column chromatography. The quantitative analyses of the various fractions, shown in Table III, show a net decrease in ester cholesterol and in triglycerides as a consequence of reaction of H_2O_2 with lipoproteins. Phospholipids were little affected. The cholesteryl esters and triglycerides of the high density lipoproteins were reduced more than in the low density lipoproteins during comparable reaction times. There appeared to be an increase in free cholesterol at the expense of cholesteryl ester in all except the markedly degraded high density lipoproteins after 4 hr reaction.

To clarify this apparent hydrolysis of cholesteryl ester, cholesterol was determined by the $FeCl_3$ method (16) applied directly to the column eluates as well as to the digitonin precipitates (17) obtained from the same column eluates after being hydrolyzed (to measure

TABLE III

Quantitative Analyses of Lipids Extracted From Peroxidized and Control Aliquots of Isolated Lipoproteins

	Cholesteryl esters		Free cholesterol		Triglycerides		Phospholipid phosphorus	
Reaction time[a] hr	Control levels, range mg/100cc	$100 \times \frac{P}{C} \pm SD$	Control levels, range mg/100cc	$100 \times \frac{P}{C} \pm SD$	Control levels, range mg/100cc	$100 \times \frac{P}{C} \pm SD$	Control levels, range mg/100cc	$100 \times \frac{P}{C} \pm SD$
Low density lipoproteins								
1-2 n = 5	33-113	53 ± 32	15.0-41.7	160 ± 45	24.0-111.0	79 ± 21	1.34-4.50	95 ± 6
4 n = 3	101-116	91 ± 1	37.6-42.9	118 ± 10	29.0-103	100 ± 2	3.43-4.51	100 ± 1
72 n = 3	87-114	24 ± 6	35.0-56.3	167 ± 20	26.8-93.0	42 ± 19	2.76-3.98	91 ± 2
High density lipoproteins								
1-2 n = 6	14.3-41.3	20 ± 8	6.9-14.2	151 ± 81	3.4-9.1	52 ± 21	1.26-3.14	87 ± 8
4	11.9-17.2	14 ± 1	2.8-5.6	32 ± 23	2.4-4.9	58 ± 15	1.22-1.44	108 ± 5

[a] See footnotes on Table I. Values are mean ± SD of number of experiments indicated by (n). Conditions of peroxide treatment are described in legend of Table I. Reaction times are the times that elapsed after addition of H_2O_2 before extraction with chloroform-methanol (2:1) was commenced.

TABLE IV
Fatty Acid Composition of Lipoprotein Associated Lipids

Fatty Acid[a]	Cholesteryl Ester				Phospholipid				Triglyceride		Free-Cholesterol[b]			
	LDL		HDL		LDL		HDL		LDL		LDL		HDL	
	C	P	C	P	C	P	C	P	C	P	C	P	C	P
Concentrations by area per cent														
16:0	11.6	12.7	18.0	45.4	31.2	34.1	41.7	39.6	31.1	29.9	29.5	26.0	31.3	38.5
16:1	3.6	4.4	6.1	10.3	1.6	1.8	3.1	6.0	3.9	4.6	7.8	5.7	5.7	7.2
18:0	0.8	1.0	1.6	9.9	14.3	15.3	19.0	13.2	4.7	4.6	8.1	7.7	10.7	19.6
18.1	20.5	22.0	27.7	25.3	11.9	11.8	15.7	10.6	38.9	34.5	27.6	31.4	25.0	14.8
18:2	51.9	50.4	39.7	—	20.0	19.4	13.8	—	13.0	12.6	12.9	16.1	6.8	0.8
20:4	8.4	6.9	3.5	—	10.6	9.1	1.6	—	—	—	2.1	3.8	—	—
Concentrations by mg/100cc[c]														
16:0	12.2	12.3	3.1	1.1	1.1	1.2	0.6	0.6	9.0	8.6				
16:1	3.8	4.3	1.1	0.3	0.1	0.1	0.0	0.1	1.1	1.3				
18:0	0.8	0.9	0.3	0.2	0.5	0.5	0.3	0.2	1.4	1.3				
18:1	21.5	21.4	4.8	0.6	0.4	0.4	0.2	0.2	11.3	9.9				
18.2	54.5	48.9	6.8	—	0.7	0.7	0.2	—	3.8	3.6				
20.4	8.8	6.7	0.6	—	0.4	0.3	0.0	—	—	—				

[a] Identification of fatty acids by retention times is only tentative because of possible modification of fatty acids by H_2O_2.

[b] Includes free fatty acids, di- and monoglycerides, and modified cholesteryl esters.

[c] Total fatty acid content of the lipid extract was determined by titration of a saponified aliquot. Fatty acid composition (by area per cent) was determined in another aliquot analyzed by GLC. Total fatty acid content in mg/100cc was multiplied by area per cent concentration for each fatty acid to compute mg/100cc concentration of each individual fatty acid.

C = control sample; P = H_2O_2 treated sample. LDL = low density lipoprotein; HDL = high density lipoprotein.

total cholesterol) or not hydrolyzed (to measure free cholesterol).

This increase in the free cholesterol eluate was detected by the $FeCl_3$ method applied both to the column eluate and to the digitonin precipitate obtained after saponification of the column eluate. However, the cholesterol precipitated by digitonin from the free cholesterol eluate from the column showed two significant relationships. (a) In the control sample the cholesterol precipitated by digitonin was the same before (112 mg/100cc) and after (112 mg/100cc) saponification. The cholesterol eluted in this fraction, therefore, was all nonesterified. Furthermore, the amount precipitated was statistically not different from that measured by the $FeCl_3$ procedure applied to the eluate (114 mg/100cc) without precipitation with digitonin. This finding demonstrates that in the column chromatography of normal serum lipids, the free and ester cholesterol were cleanly separated. (b) In the free cholesterol fraction of the peroxide-treated lipoprotein extract, the concentration of cholesterol precipitated by digitonin (114 mg/100cc) was the same as in the normal sample. This agreement shows that the levels of free cholesterol were the same in these samples. However, saponification followed by digitonin precipitation yielded a higher cholesterol level (126 mg/100cc) in the peroxidized sample. Evidently the peroxide had attacked some cholesteryl esters and produced more polar derivatives, which were eluted from the column with the free cholesterol. The cholesterol in these escaped precipitation by digitonin when hydrolysis was omitted, but was precipitated by digitonin after hydrolysis. The increase in cholesterol (14 mg/100cc) concentration due to these peroxidized esters in the free fraction was slightly less than the decrease in concentration (25 mg/100cc) in the ester fraction but the agreement was satisfactory. The results of two additional experiments have substantiated these relationships.

With the high density lipoproteins, loss of cholesterol was greater than with low density lipoproteins. This fact is further evidence that the high density lipoproteins were the more susceptible to degradation by H_2O_2. In the unfractionated extract of these lipoproteins, the concentrations of cholesterol measured by the $FeCl_3$ method applied directly to the extract were higher than the level measured after hydrolysis and precipitation by digitonin. Nevertheless, the cholesterol loss, indicated by the differences between the levels in control and peroxidized samples, was the same by both procedures. This loss was approximately equal to the sum of the losses in cholesteryl ester and free fractions eluted from the column. In contrast with the less drastic effects with the

low density lipoproteins, the peroxidized cholesteryl esters from high density lipoprotein were not eluted with the free cholesterol fraction. Probably the degraded esters were so polar that they remained on the column and thereby escaped detection while free cholesterol was eluted. Essentially these same relationships were found in a replicate experiment with another high density lipoprotein sample.

In some experiments, gas chromatography of the methylated fatty acids present in the fractions eluted from the columns was carried out. The results are presented in Table IV. As would be predicted, the highly unsaturated fatty acids were most affected by H_2O_2. The per cent loss was greatest with arachidonate and decreased as the number of double bonds decreased until, with the fully saturated C_{16} and C_{18} acids, there was no loss (compare mg/100cc of 16:0 and 18:0 in various fractions from C and P aliquots). As was observed in the quantitative studies with lipoproteins and with lipids separated on columns, the extent of loss was greatest in the high density lipoproteins.

DISCUSSION

The data presented above demonstrate that H_2O_2 added to isolated lipoproteins in vitro caused degradation of lipoproteins; the high density fraction being most extensively affected. This degradation was reflected in loss of lipids extracted from the lipoproteins. Phospholipids and triglycerides were not affected much, but cholesteryl esters were extensively peroxidized by contact with H_2O_2 for 4 hr. The observations on the cholesteryl esters indicated that H_2O_2 caused formation of derivatives (hydroperoxides of fatty acids or modified cholesterol?) which required a more polar solvent to bring about their elution from silicic acid columns. When the lipid extracts of peroxidized lipoproteins were separated by chromatography over silicic acid and the eluates analyzed by GLC, the loss of fatty acids, listed in order of decreasing loss, was arachidonic > linoleic > oleic > stearic and palmitic. In fact, there was little or no loss of stearic or palmitic acid even under conditions where all arachidonic and linoleic acid were lost.

The loss of unsaturated fatty acids is consistent with the hypothesis that they were peroxidized and polymerized into ceroid or ceroid-like pigments. Since the treatment of the extracts included evaporation to dryness at one point and since at that point the peroxidized sample extract always turned brown whereas the control extract did not, the ceroid was apparently formed at that point in the peroxidized extract. This assumption is consistent with the fact that the peroxidized lipid fractions suffered selective losses of polyunsaturated fatty acids, while the saturated acids were much less affected. These facts suggest that H_2O_2 caused formation of hydroperoxides from the unsaturated fatty acids, and that these were polymerized into ceroid pigment under the conditions of evaporation of the aliquot of the filtrate. Since ceroid is not soluble in the usual lipid solvents, the peroxidized unsaturated lipids would be missing from subsequent hexane extracts of the residue.

The effects of H_2O_2 addition to serum were much less marked than the effects on solutions of isolated lipoproteins. These effects, however, were measured with the ultracentrifuge. Robinson and Nelson (18) have shown this method to be much less sensitive than absorption spectra as an indicator of lipid peroxidation. It is assumed that the differences in susceptibility of individual serum samples to degradation by H_2O_2 reflect the fact that serum contains proteins, glutathione, ascorbic acid and tocopherols. This assumption is supported by the results of preliminary experiments which indicate that addition of ascorbic acid to isolated lipoproteins protect them from degradation by H_2O_2, as previously reported by Ray et al. (10).

Since the fat soluble vitamins are associated with the lipoproteins (19), their protective action should not be lost in the isolated lipoproteins. However, since the low density lipoproteins were isolated before the high density lipoproteins, the latter may have been deficient in this biological antioxidant. If so, the greater degradation effected by peroxide treatment of the high density fraction would be expected.

The experimental data show that in vitro H_2O_2 is capable of effecting changes in unsaturated lipids and a decrease in the mutual affinity of protein and certain lipids, particularly cholesterol esters. With isolated lipoproteins at 23 C. these effects were apparent, but with serum they were minimal. The question therefore arises whether this process occurs in vivo and causes loss of affinity between lipid and protein components of the serum lipoproteins. If so, it could be a mechanism in plaque formation in vivo. If excess peroxide does indeed play a role in vivo, this peroxide would have to act on serum lipoproteins in order to account for the separation of cholesterol from the

serum lipoprotein complex. The most likely places for such a reaction would appear to be where the oxygen tension is highest—in the lungs, in the blood soon after it leaves the lungs, or in the cells lining the arterial vasculature. The separation of lipid from lipoprotein could then deposit lipid in or on the vasculature. Such a mechanism is consistent with the observation that atherosclerosis is almost exclusively a phenomenon of the arterial system and is rarely found in the venous system. Various other lines of evidence suggest an association of atherosclerosis with lipid peroxidation. The finding of lipid peroxides and ceroid pigment in atherosclerotic plaques has been cited above. The protective effect of administration of peroxidase reported by Caravaca et al. (8) could be interpreted to mean that peroxide destruction helped prevent atherosclerosis.

Evidence provided by Cohen and Hochstein (20) shows that the primary mechanism for the reduction of H_2O_2 in erythrocytes utilizes reduced glutathione and is linked to glucose metabolism through NADPH (DPNH) produced in the hexosemonophosphate shunt. The supply of reduced cofactor, NADPH, was therefore critical to maintenance of the supply of reduced glutathione, which in turn functioned as a cofactor in the reduction of H_2O_2. Consequently, if H_2O_2 accumulation were to cause lipoprotein oxidation in vivo, this effect should be exacerbated under conditions which reduce the supply of NADPH. In this connection it may be significant that Kirk (21) found a decrease in glucose-6-phosphate dehydrogenase activity of arteriosclerotic vs. normal tissue in both aorta and coronary artery. Activity of this enzyme is inhibited in liver and red blood cells by long chain fatty acid CoA derivatives (22) and in the liver by diabetes (22) and fat feeding (23). The level of long chain acyl CoA derivatives is elevated in both heart and liver of rats by alloxan diabetes or fat feeding (24). Since both diabetes and a high fat diet are associated with increased risk of atherosclerotic heart disease, the tentative working hypothesis is entertained that in vivo a relative excess of peroxide, probably linked to a relative deficiency in NADPH, may cause oxidative changes in lipoproteins, decrease lipid-protein affinity and thereby contribute to the development of atherosclerosis.

While much more experimental evidence is required to evaluate the role of this postulated mechanism in vivo, the results of in vitro peroxidation studies reported above are compatible with the hypothesis.

ACKNOWLEDGMENTS

Technical assistance was given by Anthony E. Bowens and King Evans with the lipoprotein analyses and by Robert D. Stocks with the lipid analyses.

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[Received June 10, 1968]

Characterization of Naturally Occurring α-Hydroxylinolenic Acid[1]

C. R. SMITH, JR. and **I. A. WOLFF,** Northern Regional Research Laboratory,[2]
Peoria, Illinois 61604

ABSTRACT

The seed oil of *Thymus vulgaris* L. (Labiatae) contains 13% of a new unsaturated hydroxy fatty acid which has been characterized as α-hydroxylinolenic acid. This oil also contains the previously unknown norlinolenic (all-*cis*-8,11,14-heptadecatrienoic) acid (2%) and linolenic acid (55%). The co-occurrence of these three acids suggests that the C_{17} acid is biosynthesized by α-oxidation of linolenic acid.

INTRODUCTION

During a survey of seed oils of the Labiatae (*mint family*), Hagemann et al. (1) detected an unfamiliar hydroxy acid in oils of three *Thymus* species. According to their GLC data, the methyl ester of this acid had equivalent chain length (ECL) values of 18.7 (Apiezon L) and 23.8 (Resoflex). These ECL values led Hagemann et al. to suggest a C_{17}-hydroxy acid on the basis of the parameters of Miwa et al. (2). Apparently no such acid had been described in the literature. The present paper describes the isolation and characterization of the new hydroxy acid from *Thymus vulgaris* seed oil and shows that it is an 18-carbon compound.

T. vulgaris (common thyme) is a small shrub, native to the Old World. It has been used as a garden ornament and as a sweet herb in cookery (3).

EXPERIMENTAL PROCEDURES AND RESULTS

General Methods

Esterifications and transesterifications were carried out as follows. Samples were refluxed 1 hr in a large excess of methanol containing 1% sulfuric acid (v/v). In each case, resulting mixtures were diluted with water, chilled in an ice bath, and then extracted repeatedly with petroleum ether (bp 30-60 C). Combined extracts were dried over sodium sulfate and evaporated in vacuo. GLC analyses were performed as described by Miwa et al. (2).

Analytical TLC was performed on plates coated with Silica Gel G. The solvent system hexane-diethyl ether-acetic acid (70:30:1) was used. Spots were visualized by charring the plates with sulfuric acid-chromic acid, or by viewing them under ultraviolet (UV) light after spraying with dichlorofluorescein. For preparative TLC work, layers 1 mm thick were used.

Infrared (IR) spectra were determined with a Perkin-Elmer Model 137 or Model 337 instrument on 1% solutions in carbon tetrachloride unless otherwise specified. Optical rotatory dispersion (ORD) curves were recorded with a Cary Model 60 spectropolarimeter. Nuclear magnetic resonance (NMR) spectra were obtained with a Varian HA-100 spectrometer on 1% deuteriochloroform solutions. Melting points were determined with a Fisher-Johns block and are uncorrected.

Preparation of Mixed Methyl Esters

Coarsely ground seeds of *T. vulgaris* L. (41.4 g) were extracted overnight in a Soxhlet apparatus with petroleum ether (bp 30-60 C). Upon evaporation of solvent, 11.9 g of oil was obtained. The residual meal was dried, ground finely and reextracted. An additional 1.21 g of oil was thus obtained.

A 12.0 g portion of the oil was converted to a mixture of methyl esters by transesterification. According to GLC analysis, these esters had the following composition[3] (expressed as area per cent): C_{16}S, 4.8; C_{17}III, 2.1; C_{18} S, 1.8; C_{18}I, 7.7; C_{18}II, 12.4; C_{18}III, 57.4; and hydroxy ester, 13.3. The hydroxy ester had equivalent chain length (ECL) values of 18.7 (Apiezon L column) and 23.8 (Resoflex column). The component designated C_{17}III had ECL values of 16.6 (Apiezon L) and 18.8 (Resoflex). TLC analysis of these esters revealed a component with approximately the same R_f as methyl ricinoleate.

Countercurrent Distribution of Methyl Esters

Countercurrent distribution (CCD) of *T. vulgaris* mixed methyl esters was carried out with an acetonitrile-hexane system by the general procedure of Scholfield et al. (4). A 10.8 g portion of the mixed esters was distributed among the first 4 tubes of a 200-tube Craig-

[1]Presented at the AOCS-AACC Joint Meeting, Washington, D. C., April 1968.

[2]No. Utiliz. Res. Dev. Div., ARS, USDA.

[3]In this context, S = saturated, I = one double bond, II = two double bonds, III = three double bonds.

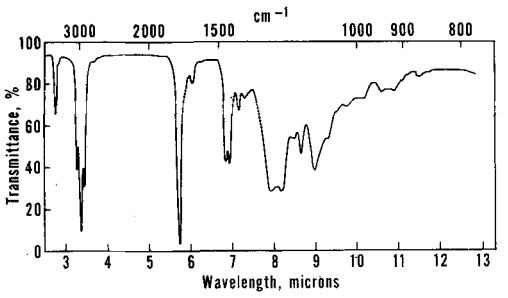

FIG. I. IR spectrum of methyl α-hydroxylinolenate (Ib).

Post apparatus. Ten milliliters of upper phase and 40 ml of lower phase were used throughout the distribution. After the 200 fundamental transfers had been completed, upper phases were decanted into a fraction collector; two transfers were combined per tube in this collector. A total of 700 transfers was applied.

The hydroxy ester (Ib), a colorless liquid, was concentrated in tubes 60 to 110 of the fundamental tubes of the machine with the peak at tube 85; GLC analyses indicated that it was obtained at least 99% pure. The IR spectrum of Ib appears in Fig. 1. Its NMR spectrum is summarized in Table I and reproduced in Fig. 2, and its ORD curve is seen in Fig 3.

The C_{17}- and C_{18}-trienes were isolated from appropriate tubes in the decant series; pertinent data concerning selected tubes are summarized in Table II. The C_{17}-triene (VIII) was obtained as a colorless liquid; its NMR spectrum is summarized in Table I. The IR spectrum of VIII showed neither *trans* C=C absorption (965 cm^{-1}) nor hydroxyl absorption.

TABLE I

NMR Spectra of Methyl 2-Hydroxy-*cis*-9,*cis*-12,*cis*-15-Octadecatrienoate (Ib) and Methyl *cis*-8,*cis*-11,*cis*-14-Heptadecatrienoate (III)

Assignment	τ-Value, ppm	Number of protons Ib	III
CH_3, β to double bond (triplet)	9.04	3	3
CH_2, shielded (singlet)	8.68	10	8
CH_2, α to double bond	7.96	4	4
CH_2, α to carboxyl (triplet)	7.71	....	2
CH_2, diallylic (apparent triplet)	7.26	4	4
OCH_3 (singlet)	6.3–6.4	3	3
CH-OH, proton attached to carbon, α to carboxyl (apparent singlet)	5.97	1	....
Olefinic H (multiplet)	4.6–4.7	6	6
Total protons		31[a]	30

[a] Does not include hydroxyl proton.

Catalytic Hydrogenation of Unsaturated Hydroxy Ester (Ib)

Ester Ib (0.190 g) was hydrogenated at ambient temperature and atmospheric pressure in 50 ml of methanol with platinum oxide catalyst. The saturated product (II), 0.184 g, mp 51-52 C, was recrystallized from hexane. The product (0.079 g) had mp 52-53 C and ECL values of 19.2 (Apiezon L) and 22.3 (Resoflex). The IR spectrum of II was very similar to that of Ib, except in the C-H stretching region. The ORD curve of II had the following characteristics: $[\alpha]_{589}^{25}$ –2.1°, $[\alpha]_{550}$ –3.2°, $[\alpha]_{500}$ –4.3°, $[\alpha]_{400}$ –9.8°, $[\alpha]_{350}$ –18.8°, $[\alpha]_{300}$ –41.2° (c. 0.93, chloroform).

Reductive Deoxygenation of Saturated Hydroxy Ester (II)

Saturated ester II (70.0 mg) was refluxed 24 hr with 6 ml of 48% hydriodic acid and 50 mg of red phosphorus. The mixture was diluted with water and extracted repeatedly with petroleum ether. After having been dried with sodium sulfate, the combined extracts were evaporated. The oily residue was refluxed 1 hr with 5 ml of methanol, 1 ml of concentrated hydrochloric acid and 0.20 g of granulated zinc. Evaporation of extracts provided a product (23.3 mg) that was shown by GLC analysis to be essentially pure methyl stearate (III).

Lithium Aluminum Hydride Reduction of Saturated Hydroxy Ester (II)

The saturated hydroxy ester II (45.0 mg) was reduced with excess lithium aluminum hydride in ethyl ether. Excess hydride was destroyed with wet ether and precipitated matter was brought into solution with hydrochloric acid. The organic layer afforded 31.5 mg of

TABLE II

Composition of Selected CCD Fractions (Determined by GLC and expressed as area percent)

Transfers completed	Weight, mg	% C_{17}III	% C_{18}III
400	6.0		
420	21.1		
440	80.5		
460	150.7		
480	145.1		
500	86.1		
520	36.1		
540	15.0	14.3	85.7
560	7.7	78.2	21.2
580	5.1	89.5	10.1
602	2.0	85.0	8.5
620	1.2	33.3	16.7
640	0.8		

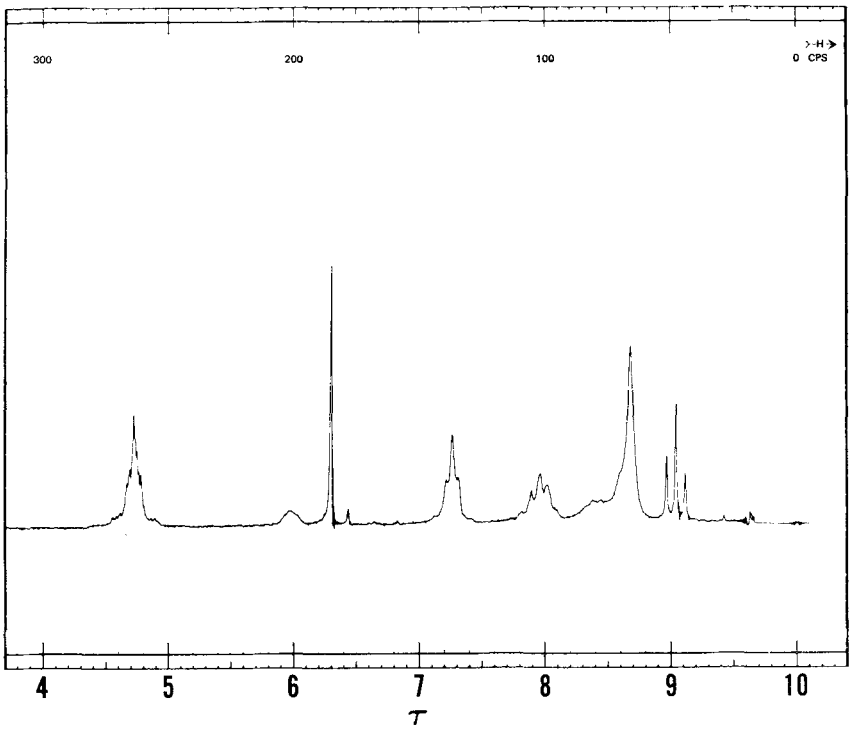

FIG. 2. 100 MHz. NMR spectrum of methyl α-hydroxylinolenate (Ib).

amorphous, glassy material (IV). Attempts to crystallize IV were without success.

Permanganate-Periodate Oxidations

Oxidative cleavages were carried out by von Rudloff's permanganate-periodate method, specifically the modification in which *t*-butyl alcohol is used as a cosolvent (5).

Hydroxy Ester (Ib). A 40.0 mg portion of Ib yielded acidic cleavage fragments, which were analyzed *per se* by GLC. Propionic acid (VI) was the major monocarboxylic acid detected. The cleavage products were converted to methyl esters (13.0 mg) which were analyzed by GLC. The major cleavage product (VII) identified had ECL values of 12.7 (Apiezon L) and 20.5 (Resoflex).

1,2-Diol (IV). Oxidation products derived from IV were converted to methyl esters and analyzed by GLC. Heptadecanoic acid (V) was revealed as the major cleavage product isolated; small amounts of $C_{16}S$ and of some unidentified components also were noted.

C_{17}-Triene (VIII). A 22.1 mg portion of VIII, 85–90% pure by GLC, was oxidized to acidic cleavage products, which were analyzed by GLC. Propionic acid was the principal fragment identified. The acidic cleavage products were converted to methyl esters. According to GLC analyses, the esterified product was 82.7% octanedioate and 15.3% nonanedioate.

Hydrogenation of C_{17}-Triene

Ester VIII, 90% C_{17}III by GLC, was hydrogenated essentially as described for Ib. The product, a low melting solid, was 91.2% methyl heptadecanoate.

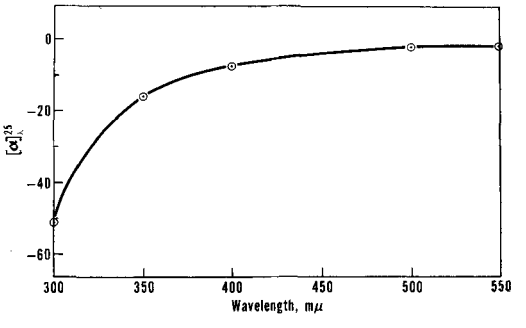

FIG. 3. ORD curve of methyl α-hydroxylinolenate (Ib) (c. 4.5, methanol).

Chromium Trioxide Oxidation of Saturated Hydroxy Ester (II)

Ester II was oxidized by the method of Boast and Polgar (6), except that a longer reaction time was used. II (114 mg) was dissolved in 2 ml of glacial acetic acid and was treated with a solution of 77.5 mg of chromium trioxide in 1 ml of acetic acid-water (1:1). The mixture was heated 1 hr at 65 ± 5 C, then poured into ice water and extracted repeatedly with petroleum ether. The combined extracts were dried over sodium sulfate. The product (101 mg) was obtained by evaporating the solvent in vacuo. The IR spectrum of this product showed maxima at 1710, 1730 and 1750 cm^{-1} as well as features in the carbon-hydrogen stretching region that indicated free carboxylic acid. This product was subjected to preparative TLC. Two bands were visualized and were removed from the plate with a Goldrick-Hirsch aspirator. Samples were eluted from the respective silica bands with ethyl ether. From the slower moving band was obtained 15.7 mg, 62.6% $C_{17}S$ and 34.5% $C_{16}S$ as shown by GLC of the corresponding methyl esters. The faster moving of the two bands (44.2 mg) had IR maxima at 1740, 1760 and 1780 cm^{-1}.

Sodium Borohydride Reduction of Fraction From Preparative TLC

The faster moving of the two TLC fractions from oxidation of II (preceding paragraph) was dissolved in 10 ml of methanol. Sodium borohydride (0.15 g) suspended in 5 ml of methanol was added and the mixture was refluxed 2 hr, then chilled, acidified with hydrochloric acid, and extracted repeatedly with petroleum ether. Upon evaporation of solvent, 29.6 mg of material was obtained which was treated under esterification conditions with 1% sulfuric acid in methanol. The product thus provided was chromatographed on a preparative silica plate. Two bands were visualized and were eluted from the silica with ethyl ether. The faster moving of these two bands yielded 3.7 mg, mp 60-64 C, whose IR spectrum was identical with that of II. The slower moving fraction yielded 7.8 mg whose IR spectrum (in $CHCl_3$) had strong hydroxyl absorption at 3590 and 3630 cm^{-1}, but no carbonyl peak. This material was not characterized further.

DISCUSSION

When analyzed by GLC, the methyl ester (Ib) of the hydroxy acid from *T. vulgaris* seed oil had ECL values such that Hagemann et al. (1) suggested a C_{17} structure. Similarly, the ECL values for II, our hydrogenation product from Ib, seemed too low for a C_{18} hydroxy ester. However, when Ib was hydrogenated and then reduced with hydrogen iodide-phosphorous, the product was methyl stearate (III). Thus, it was proved that Ia and II have a normal C_{18} carbon skeleton. Tulloch (7) compared the GLC retention times of the entire series of isomeric methyl hydroxyoctadecanoates on both polar and nonpolar liquid phases. He found that the 2 isomer had shorter retention times than any other of the series, especially on a polar column (ethylene glycol succinate). In the light of his data, the observed ECL values were consistent with an α-hydroxy ester structure for Ib and II.

The IR and NMR spectra of Ib (Fig. 1 and 2) and of its hydrogenation product (II) likewise indicated an α-hydroxy ester structure. The IR spectra of Ib and II have pronounced maxima at 1110, 1210, and 1265 cm^{-1} that are in accord with those of known α-hydroxy esters, but differ markedly from IR spectra of "typical" methyl hydroxy esters, e.g., methyl ricinoleate. The hydroxyl absorption which occurs as a single, sharp peak at 3550 cm^{-1} is distinctive. The NMR spectra of Ib and I showed a signal at τ5.97 (broad singlet), associated with the proton attached to carbon in a CHOH group α to a carboxyl (8), but lacked the triplet at τ7.7, characteristic of a CH_2 group α to a carboxyl (9). Both IR and NMR spectra of Ib have features that indicate the presence of a series of *cis*-double bonds with methylene interrupted spacing. In the IR spectrum there is no maximum at 965 cm^{-1} that would indicate a *trans* double bond. The NMR spectrum (Table I) shows four protons in methylene groups flanked by double bonds (τ7.26).

Catalytic hydrogenation of Ib provided saturated methyl ester II. Failure of Ib to undergo oxidative cleavage at the α carbon with permanganate-periodate is not entirely unexpected since α-hydroxy acids are oxidized only very slowly by periodic acid (10). In contrast, ester IV, the lithium aluminum hydride reduction product of II, was readily degraded by permanganate-periodate. Heptadecanoic acid (V) was the major isolable cleavage product. This transformation further supported the α-hydroxy formulation for Ia.

Oxidation of Ib with permanganate-periodate afforded propionic acid (VI) and α-hydroxynonanedioic half ester (VII). These cleavage products place double bonds at positions 9 and 15. Since the sharp triplet centered at τ9.04 is characteristic of a methyl group β to

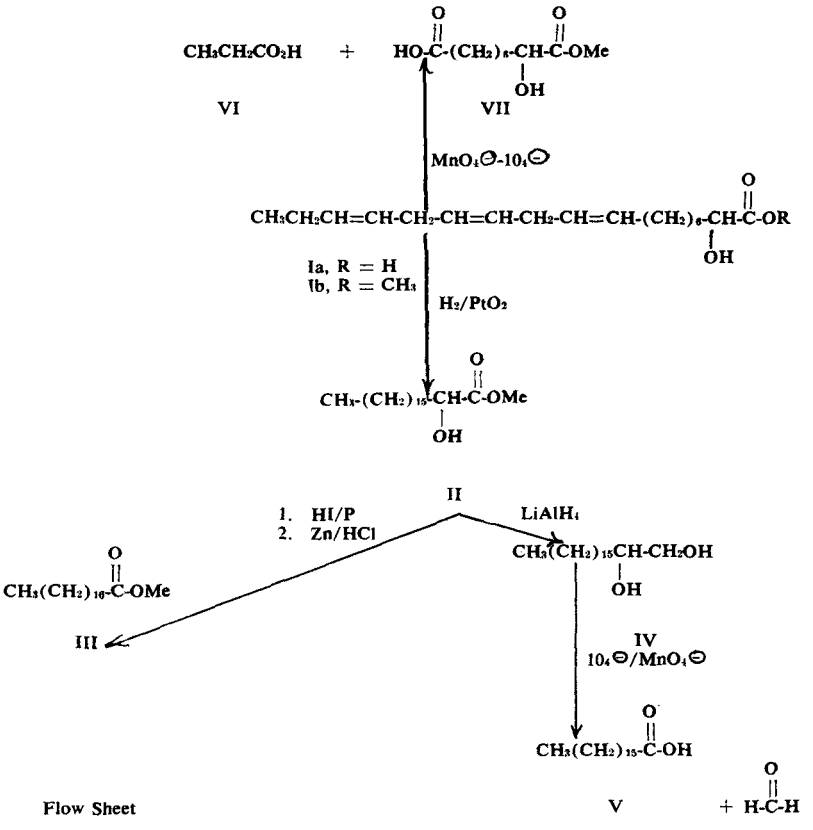

FIG. 4

a double bond, as in methyl linolenate, the NMR spectrum of Ib likewise indicates a double bond in the 15 position. The apparent triplet at τ7.96, which has an area equivalent to four protons, established the presence of two methylene groups flanked by double bonds. Consequently, Ib is a 9,12,15-triene and Ia may be termed α-hydroxylinolenic acid.

Unsaturated ester Ib in methanol solution showed a plain negative ORD curve, as did its saturated counterpart (II) in chloroform solution. Apparently, the rotation of an optically active methyl 2-hydroxyoctadecanoate has not been recorded in the literature, except in a paper by Horn et al. (11). Even in this case, their compound (derived from wool wax) was mixed with a branched-chain isomer. However, Horn et al (11,12) isolated pure, optically active methyl esters of 2-hydroxytetradecanoic and 2-hydroxyhexadecanoic acids; the observed rotation for each was $[\alpha]_D$ −3.6° in chloroform, a value close to that of II. On this basis, we make a strictly tentative assignment of the D-configuration to Ia and Ib. Morris and Hall (13) recently assigned the D-configuration to naturally occurring 2-hydroxysterculic acid on similar grounds. The assignment of absolute configuration to compounds with weak optical activity can be hazardous, since the sign of rotation may not be the same in different solvents (12,14–16). The rotations of some of these compounds show a concentration dependence such that they may change *sign* even with the same solvent (15).

GLC analyses of the mixed methyl esters of *T. vulgaris* oil indicated the presence of 2% of an unfamiliar component with ECL values that were compatible with a C_{17} homolog of methyl linolenate. Such an ester was indeed isolated from appropriate CCD fractions. Because of its shorter chain length, a C_{17}-triene would be expected to be slightly less mobile in the hexane-acetonitrile solvent system than linolenate. In actual practice, the peak for linolenate occurred at 470 transfers, and for the C_{17}-triene at 560 transfers.

Hydrogenation of the C_{17}-triene (VIII) produced methyl heptadecanoate and thus demon-

strated that VIII has a normal C_{17}-skeleton. Permanganate-periodate oxidation of VIII yielded propionic acid and octanedioic acid half ester. These observations, together with the IR and NMR spectra of VIII, supported the conclusion that VIII is the methyl ester of all-*cis*-8,11,14-heptadecatrienoic acid. Particularly important features of the NMR spectrum of VIII were the sharp triplet at τ9.04 (3 protons, methyl group β to double bond), and the triplet at τ7.26 (4 protons, methylene groups flanked by double bonds). VIII may be termed "norlinolenic" acid to emphasize its relationship to linolenic.

α-Oxidation of fatty acids in higher plants is now a well established biochemical process (17). Working with solutions of dried pea-leaf powder, Hitchcock et al. (18) have just demonstrated that the preferred intermediate α-hydroxy acid has the L-configuration. The corresponding D-isomer also is formed, but is not readily degraded by enzymes in this system and, consequently, accumulates. In contrast, the L-isomer disappears rapidly as the α-oxidation reaction proceeds (19). Although α-hydroxy acids occur generally as components of cerebrosides and other sphingolipids, only recently have any been found as a glyceride constituent in a seed oil. Morris and Hall (13) isolated appreciable amounts of 2-hydroxysterculic acid from seed oils of *Pachira insignis* and *Bombacopsis glabra*. These workers pointed out that if malvalic acid is formed by α-oxidation of sterculic, an α-hydroxy acid would be expected as a biosynthetic intermediate. Although sterculic and malvalic acids commonly occur together, such an intermediate had not been detected previously. The co-occurrence of linolenic, α-hydroxylinolenic and norlinolenic acids in *Thymus* seed oil suggests an analogous degradation which involves Ia (or its optical antipode) as an intermediate.

To our knowledge, the melting point of an optically active methyl 2-hydroxyoctadecanoate has not been reported. Our optically active sample of II has mp 52-53 C, substantially below the value reported for the corresponding racemate, mp 65.7-65.9 C (20). In order to clarify these melting point relationships, steps were taken to convert II to a racemate by an oxidation-reduction sequence. II was oxidized with chromium trioxide in acetic acid, and the resulting oxidation product was reduced with sodium borohydride The oxidation step was not straightforward and products had to be purified by preparative TLC at two stages in the reaction sequence. Although the overall yield was low, a dramatic increase in melting point was demonstrated since the racemic ester isolated had mp 60-64 C.

ACKNOWLEDGMENTS

GLC analyses by J. M. Hagemann; NMR spectra by D. Weisleder; ORD curves by K. L. Mikolajczak; seeds furnished by Q. Jones, USDA, Crops Research Division, Beltsville, Md.

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[Received June 10, 1968]

Cis-11-Hexadecenoic Acid From *Cytophaga hutchinsonii* Lipids[1]

ROBERT W. WALKER, Institute of Agricultural and Industrial Microbiology, Department of Environmental Sciences, University of Massachusetts, Amherst, 01002

ABSTRACT

The principal fatty acid (90+%) of the monoene fraction of *Cytophaga hutchinsonii* has been identified as *cis*-11-hexadecenoic acid. This fatty acid, which constitutes 29% of the total lipid fatty acids, is located principally in the phosphatidyl ethanolamine fraction.

INTRODUCTION

THE LIPIDS OF THE MYXOBACTERIA have been little investigated (1,2). During the course of studies characterizing the lipids of *Cytophaga hutchinsonii* (2), a novel unsaturated fatty acid was found. This communication describes the characterization of this fatty acid.

METHODS

Growth of Cytophaga hutchinsonii

Cytophaga hutchinsonii, obtained from Katherine Middleton, San Francisco State College, was cultivated in a salts, glucose medium (Table I). The medium was dispensed in 3 liter Fernbach flasks and sterilized. Sterile glucose and a 10% inoculum of rapidly growing cells in growth medium were added to the sterile medium. Flasks were incubated at 27 C for four to five days on a rotary shaker (G-52 New Brunswick Scientific Co., N. J.). Cells were harvested by centrifugation and stored frozen at -20 C until lyophilized.

Extraction

Freeze-dried cells (40 g) were extracted with 500 ml chloroform-methanol (2:1 v/v) with shaking at room temperature for 2 hr (2x), for 3 hr (1x) and for 18 hr (1x). The residue was then extracted with 600 ml chloroform-methanol 7:1 (v/v)—7% ammonium hydroxide (sp gr 0.9) with shaking for 4 hr.

Isolation of Fatty Acids

The crude lipid extract (2.56 g) was refluxed 2 hr with 220 ml methanol-benzene-H_2SO_4 (conc) (20:10:1 v/v/v). After removal of a portion of the solvents by evaporation, the methyl esters were extracted with petroleum ether (bp 37-51 C) and washed twice with distilled water and once with 0.1M $NaHCO_3$. The crude methyl ester preparation obtained (0.8 g) was streaked on silver nitrate impregnated silica gel plates (Adsorbosil 1, Applied Science Labs. Inc., State College, Pa., slurried in 5% $AgNO_3$, spread to a thickness of 0.5 mm, activated at 120 C for 45 min). The plates were developed with petroleum ether-diethyl ether (85:15 v/v) and then sprayed with a dilute solution of fluorescein. The monoene fraction, located with an ultraviolet light, was scraped from the plates and eluted from the adsorbent with diethyl ether.

The C16 monoene (94% of the monoene fraction) was purified to 99% purity by preparative GLC. (An A90-P3 instrument was used Varian Aerograph, Walnut Creek, Calif.) equipped with a 5 ft × ¼ in. column, Chromosorb W, 60-80, 20% SE-30, at 210 C, helium flow 120 ml/min.

Location of the Double Bond

The purified C16 monoene was oxidized with von Rudloff oxidant (3). Methyl esters of the cleavage products were prepared according to the method of Schlenk and Gellerman (4). Dimethyl hendecanedioate was prepared from vaccenic acid (90%) (K. & K. Labs., Inc., Plainview, NY, prepared by von Rudloff oxidation and preparative GLC) and was obtained from Lachat Chemicals, Inc., Chicago, Illinois.

TABLE I
Cultivation Medium

Medium	Amount
	g/liter
Na_2HPO_4	2.84
KH_2PO_4	2.72
$MgSO_4$	0.5
$CaCl_2$	0.01
$(NH_4)_2SO_4$	1.0
add 1 ml solution C/liter glucose[a]	
adjust to pH 7.2 with NaOH solution C	5.0
	mg/liter
$ZnSO_4 \cdot 7H_2O$	50
$FeCl_3$	30
$MnCl_2$	50
$CuSO_4$	10
$CoCl_2$	10
$(NH_4)_6MO_7O_{24} \cdot 4H_2O$	200
H_3BO_3	10

[a]Added aseptically to sterile medium.

[1]Contribution from the Agricultural Experiment Station, University of Massachusetts, Amherst.

Saturation of the Double Bond

Ten milligrams of C16 monoene were placed in a flask containing 10 ml 90% ethanol and 20 mg 5% platinum on charcoal (Matheson, Coleman and Bell, Norwood, Ohio) which had been purged with H_2 for ½ hr. After a 1 hr reaction period the saturated methyl ester was separated from the catalyst and analyzed by GLC.

Isolation of Phosphatidyl Ethanolamine

Crude lipid extract (830 mg) was fractionated on 80-200 mesh silicic acid (prepared from Mallinckrodt silicic acid, Malinckrodt Chem. Works, St. Louis, Mo., 100 mesh, activated at 120 C overnight), in a 30 x 150 mm column. Chloroform (600 ml) eluted 14 mg of dark red material, acetone (1300 ml) eluted the glycolipid fraction (355 mg) and methanol (800 ml) eluted a fraction (443 mg) composed almost entirely of phosphatidyl ethanolamine (PE). This fraction, which constituted over 50% of the crude total lipid extract, was purified by preparative TLC on Adsorbosil 1 plates developed with chloroform-methanol-water (65:25:4 v/v/v). The purified PE (3.9% P, 2.5% N) was deacylated (5). The water soluble phosphate ester had R_f's identical with those of purified egg glycerophosphorylethanolamine in two solvent systems Isopropanol-ammonium hydroxide (sp gr 0.9)-water (35:9:6 v/v/v) *n*-butanol acetic acid-water (5:3:1 v/v/v):

Phospholipase A Hydrolysis of PE

PE was hydrolyzed with *Crotalus adamanteus* venom (Ross Allen's Reptile Institute, Silver Springs, Fla.) and with *Naja naja* venom (Sigma Chemical Co., St. Louis, Mo.) according to the method of Van Golde and Van Deenen (6). The *C. adamanteus* experiments were incubated overnight at 27 C with shaking, the *N. naja* experiments were incubated 6 hr at 27 C with shaking. Incomplete hydrolysis was observed with *C. adamanteus* venom at shorter incubation times. Methyl esters of total PE, lyso PE and liberated fatty acids were prepared by heating lipid with dry methanol containing 5% dry HCl (w/v) at 60 C overnight. The methyl esters, extracted with petroleum ether, were analyzed by GLC.

Gas Chromatography

Analyses were performed on an A-600 C gas chromatograph equipped with linear temperature programmer (Varian Aerograph, Walnut Creek, Calif.). The columns were a 12 ft x ⅛ ft stainless steel column packed with acid washed Celite 545 (60/80 mesh) coated with 15% diethylene glycol succinate and a 15 ft x 1/16 in. stainless steel column (Varian Aerograph, Walnut Creek, Calif.) packed with Aeropak (100/120 mesh) coated with 3% SE-30. Monocarboxylic acid esters were analyzed on the Degs column at 65 C (N_2 pressure 7 psi), dicarboxylic acid esters at 212 C (N_2 pressure 14 psi) and fatty acid esters at 187 C (N_2 pressure 14 psi). On the SE-30 column, fatty acid esters were analyzed at 200 C and with temperature programming from 200-270 C at 2 C/min (N_2 pressure 54 psi).

TABLE II

Fatty Acid Composition[a]

	Phosphatidyl Ethanolamine			Total Lipid Extract
	Total	1 position[b]	2 position[c]	
*n*14[d]	1.0	0.9	0.4	0.9
*i*15	21.1	21.0	14.0	16.6
*a*15	2.1	2.0	0.5	1.3
*n*15	1.8	2.6	0.9	1.7
*n*16	19.8	19.0	24.0	17.6
*a*17	8.0	13.0	....	25.7
16:1	45.0	37.0	59.0	29.2
*n*17	1.2	3.8	0.9	3.0
unidentified	tr[e]	0.7	0.3	4.0

[a]Tentative identification based on retention time data obtained on a polar and nonpolar column at several temperatures, as weight per cent.

[b]From lyso-PE liberated by phospholipase A hydrolysis.

[c]From free fatty acids liberated by phospholipase A hydrolysis.

[d]*n*=normal, *a*=*anteiso*, *i*=*iso*, 16:1 = hexadecenoic acid.

[e]Trace.

RESULTS AND DISCUSSION

One hundred and forty-seven grams of freeze-dried cells were obtained from 370 liter of growth medium, a yield of approximately 0.4 g/liter (dry weight). Neutral solvent extraction of 40 g of freeze-dried cells yielded 8.30 g of lipid and extraction with basic solvents yielded an additional 0.88 g of lipid for a total crude lipid yield of 9.18 g (22.9% yield from lyophilized cells).

The C16 monoene comprised 29% of the fatty acids of the total lipid extract, 45% of the purified phosphatidyl ethanolamine fraction (Table II). Most of this fatty acid was located in the PE fraction.

The infrared spectrum of the purified C16 monoene showed no unusual features and the absence of an absorption band between 960 and 970 cm^{-1} (*trans* C-H out of plane deformation) (7) indicates the double bond is of *cis* configuration. Spectra were made on a Perkin-Elmer model 331 spectrophotometer

(Perkin-Elmer Corp., Norwalk, Conn.). The sample was analyzed as the neat methyl ester between NaCl plates using a micro-beam condenser.

Gas-liquid chromatographic analysis of the *C. hutchinsonii* monoene before and after catalytic hydrogenation was performed. The retention time of the monoene after saturation was identical to that of methyl palmitate, proving a straight-chain C_{16} skeleton for the acid. Retention time relative to the retention time of methyl palmitoleate (Applied Science Labs., State College, Pa.) is 1.06.

Oxidation of the monoene with von Rudloff oxidant yielded a 5 carbon monocarboxylic acid and an 11 carbon dicarboxylic acid locating the double bond between the 11th and 12th carbons of the monoene. Thus the C-16 fatty acid monoene from *C. hutchinsonii* is *cis*-11-hexadecenoic acid (*cis* palmitvaccenic acid).

No palmitvaccenic acid was found in the glycolipid fraction. Most of this fatty acid was found in the PE fraction where it was located principally at the 2 position (Table II).

The distribution of the 16:1 fatty acid at the 2 position of the PE molecule is similar to the monoene distribution found in the PE fractions of other gram-negative bacteria. Van Golde and Van Deenen (6) found, in studies of an extracellular PE from *Escherichia coli,* that the 16:1 and 18:1 fatty acids were located principally at the 2 position.

Hildebrand and Law (8), and Lennarz (9) found the unsaturated and cyclopropane fatty acids of *Azotobacter agilis, E. coli* and *Serratia marcescens* PE to be located at the 2 position. In the case of *Clostridium butyricum* PE, however, the unsaturated and cyclopropane fatty acids were on the 1 position, and in *Agrobacterium tumescens* PE these fatty acids were randomly distributed.

Okuyama et al. (10) found the 16:1 and 18:1 fatty acids to be randomly located in PE from *Mycobacterium tuberculosis* (BCG), *M. phlei* and *M. butyricum.* Walker and Howard (11) observed essentially the same distribution in the case of PE isolated from *M. smegmatis.* In the mycobacteria, location of the saturated fatty acids and 12-methyl-stearic acid are more specific than the unsaturated acids.

Palmitvaccenic acid has been found thus far in very few organisms. M. E. de Tomas et al. (12) reported the first occurrence in nature where palmitvaccenic acid was found as a major fatty acid. This acid, present in the seed fats of *Gevuina avellana,* comprised 22% of the unsaturated fatty acids. Gunstone et al. (13) confirmed the findings of de Tomas et al. They found palmitvaccenic acid accounted for 24 mole % of the total seed fatty acids and was located principally on the 2 position of the triglycerides.

With the exception of the seed fat of *G. avellana* (12, 13), palmitvaccenic acid generally is present only as a minor portion of the hexadecenoic acid fraction. Hofmann and Tausig (14) found palmitvaccenic acid comprised about 10% of the 16:1 fraction of the fatty acids of a group C *Streptococcus* species. Korn (15) has found the 11, 12 isomer constitutes about 10% of the 16:1 acids of *Euglena gracilis.*

Gellerman and Schlenk (16) found palmitvaccenic acid to be a minor component of the fatty acids obtained from nuts of *Ginkgo biloba.*

Scheuerbrandt and Bloch (17) found palmitvaccenic acid in the 16:1 fraction of *Clostridium pasteurianum.* This acid, comprising 1% of the total fatty acids, made up about 25% of the 16:1 acids.

The presence of major amounts of palmitvaccenic acids in the lipids of *C. hutchinsonii* is of interest. This organism, which is classified apart from the true bacteria, possesses a gliding type of motility and cellular morphology differing greatly from the true bacteria. That the fatty acids of this organism differ from those of the true bacteria is therefore not surprising and may reflect the existence of different biosynthetic pathways and different orientation of these compounds in cell wall (18) and membrane.

ACKNOWLEDGMENTS

Technical assistance from G. L. Howard, partial support by the University of Massachusetts Research Council.

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[Received July 17, 1968]

Studies on Liver Phosphatidyl Cholines: I. Effects of Fatty Liver Induction on Phosphatidyl Cholines From Liver Mitochondria and Microsomes

JAMES E. MILLER and **W. E. CORNATZER,** The Guy and Bertha Ireland Research Laboratory, University of North Dakota School of Medicine, Grand Forks, North Dakota 58201

ABSTRACT

Protein, total phospholipid, phosphatidyl cholines and phosphatidyl choline fractions from liver mitochondria and microsomes of female rats were analyzed after treatment with CCl_4 (0.3 ml of CCl_4 suspended in corn oil) or ethionine (50 mg in 0.9% saline) or after feeding a choline deficient, low protein diet for seven days. Phosphatidyl cholines were separated into four fractions differing in the degree of fatty acid unsaturation. Over 50% of total phosphatidyl choline phosphorus was present in fraction 3 of liver mitochondria and microsomes. The major fatty acid in fraction 1 was docosahexaenoic acid. Fraction 4 contains oleic and linoleic acids. Arachidonic acid occurs in fraction 2 and 3. Ethionine decreased the amount of microsomal protein and phosphatidyl choline fraction 1 of mitochondria. Microsomal protein was decreased by CCl_4. The choline deficient, low protein diet caused a decrease in mitochondrial and microsomal phospholipids. The amount of the mitochondrial phosphatidyl choline decreased. Corn oil increased the level of phosphatidyl choline fraction 3. Choline deficiency decreased the amount of phosphatidyl choline fraction 3, increased fraction 4 of mitochondria and microsomes and increased fraction 1 of microsomes.

INTRODUCTION

IT HAS BEEN SHOWN that fat accumulation in the liver results from the administration of carbon tetrachloride (CCl_4), ethionine or dietary production of a choline deficiency (1,2). One of the difficulties in understanding the production of a fatty liver is the large number of mechanisms which may cause this defect. Carbon tetrachloride causes damage to the endoplasmic reticulum (3,4) within several hours after administration and later to mitochondria (5,6). Ethionine induces a fatty liver in the female rat (7), inhibits liver protein synthesis (8), and decreases fatty acid oxidation (9). The ethyl group of this compound is incorporated into the normal acceptors of the methionine methyl group (10). CCl_4 (11) and ethionine (11,12) decrease ATP levels in the liver. Administration of ATP (13) can alleviate the fat accumulation induced by these agents. Choline deficiency causes hepatic triglyceride accumulation (14), decreased fatty acid oxidation (15), and an alteration of phospholipid fatty acids without changing the concentration of phospholipid within the liver (16).

The total liver phospholipid levels are unchanged during fat accumulation but the fatty acid composition is altered by CCl_4 (17) or choline (16) deficiency. However, this does not exclude the possibility that the phosphatidyl cholines may be altered. Phosphatidyl cholines from liver microsomes and mitochondria of the female rat have been separated into fractions differing in the degree of fatty acid unsaturation and the amount of each fraction measured after treatment of female rats with CCl_4 or ethionine or a choline deficient-low protein diet.

MATERIALS AND METHODS

Protein standard albumin was obtained from Armour Pharmaceutical Company. Silica gel H was purchased from Brinkmann Instruments. DL-ethionine was obtained from the California Foundation for Biochemical Research, Los Angeles. Corn oil, Mazola, was obtained from Best Foods Division, Corn Products Company. Safflower oil was obtained from General Mills, Incorporated. Casein, cod liver oil, non-nutritive bulk, salt mixture and vitamins were purchased from the Nutritional Biochemicals Corporation. Chloroform, methanol, petroleum ether, and hexane were either nanograde or spectranalyzed reagents. Triton X-100 was obtained from Sigma Chemical Company, and 1-amino-2-naphthol-4-sulfonic acid was purchased from Matheson, Coleman and Bell. All other chemicals were analytical reagents.

Female rats of the Sprague-Dawley strain were used to study phosphatidyl choline levels during fat accumulation in the liver. Wire-bottom cages were used to house individual rats, and the rats were allowed free access to food and water. All rats were fed a standard laboratory chow (Purina Laboratory Chow) obtained from the Ralston Purina Company, St. Louis, Mo. The female rats were divided into six groups, three of which were experimental animals and three were controls. Group 1 was maintained on normal laboratory chow and received no injections. These animals served as a base for all experiments and as controls for rats fed a choline deficient, low protein diet for seven days (18). The components of the diet and fatty acids composition have been described by Glende and Cornatzer (18). Group 2 of the experimental rats received intraperitoneally 0.3 ml of CCl_4 in 0.7 ml of corn oil while a control group received corn oil. The dose of CCl_4 is similar to that reported by Maling et al. (19). These rats were killed 7 hr later. Group 3 of the experimental rats received an intraperitoneal injection of 50 mg of DL-ethionine in 0.9% NaCl (20) while a control group was given 0.9% NaCl, and these rats were killed 12 hr later.

The rats were killed by decapitation. The livers were removed, rinsed with cold water, blotted, weighed and homogenized with ice cold 0.25 M sucrose in a Potter-Elvehjem homogenizer with a teflon pestle. The mitochondrial and microsomal fractions were isolated by differential centrifugation (21). The nuclear fraction was separated from the homogenate by centrifuging for 10 min at 800 x *g*. The nuclear pellet was homogenized and the nuclear suspension centrifuged. The supernatant solutions were combined and centrifuged for 10 min at 14,500 x *g* to sediment the mitochondrial fraction. The mitochondrial pellet was washed twice. The combined supernatant solutions from the mitochondrial preparation were centrifuged at 78,450 x *g* for 45 min to sediment the microsomal pellet. Protein was determined by a modified Biuret method (22, 23). The method of Folch et al. (24) was employed to extract and purify lipids from mitochondria and microsomes. Lipids were stored in a dilute chloroform solution under dry nitrogen at –18 C. The total phospholipid phosphorus (25,26) was determined on an aliquot of the chloroform solution. Phosphatidyl cholines were isolated from the lipid extract by TLC (27). Phospholipids were identified by comparison with purified phospholipid standards. Plates were sprayed with a 0.008% rhodamine 6G solution and viewed under ultraviolet light to identify and outline the band of gel containing the phosphatidyl cholines. After the silica gel containing the phosphatidyl choline had been scraped into a flask containing 20 ml of chloroform-methanol (2:1 v/v), the phosphatidyl cholines were eluted from the gel by filtration of the chloroform-methanol solution with the aid of a sintered glass funnel (medium porosity). The gel was washed twice with chloroform-methanol-water (200:97:3) and once with methanol. Quantitative recovery of phosphatidyl choline was possible with this elution procedure. Recovery values represented 94% of the total phosphorus. The filtrate was washed with 0.2 volumes of 0.04% $CaCl_2$. A dilute solution of the phosphatidyl choline extract in $CHCl_3$ was stored under dry nitrogen at –18 C. On an aliquot of the chloroform solution the total phosphatidyl choline phosphorus (25,26) was determined and the percentage of total lecithin phosphorus per total phospholipid phosphorus calculated. Separation of the phosphatidyl choline fractions was carried out, the TLC on silica get H impregnated with silver nitrate (28). The phosphatidyl choline fractions were identified by spraying with a 0.01% methanolic solution of 2,7-dichlorofluorescein and under ultraviolet light (28). Each phosphatidyl choline fraction gave a positive test for choline (29).

Phosphorus was determined by the method of Fiske and Subbarow (26) after elution of the phosphatidyl choline from silica gel or silica gel impregnated with silver nitrate by the following series of solvents: Chloroform-Methanol (2:1 v/v); Chloroform-Methanol-Water (200:97:3 v/v/v); Methanol-water (97:3 v/v); and Methanol. Recovery values represented 98% of the phosphorus applied to silica gel impregnated with silver nitrate. The percentage of total lecithin phosphorus in each phosphatidyl choline fraction is similar to that reported (30).

Methyl esters of the phosphatidyl choline fatty acids were prepared by the method of Morgan et al. (31). Fatty acids were analyzed using a Barber-Colman Model 10 Gas Chromatograph with a ^{90}Sr detector as described by Glende and Cornatzer (18). Identification of the methyl ester derivatives of the fatty acids were made by comparing the retention ratios (relative to methyl palmitate) to those obtained with standards (Applied Science Laboratories

TABLE I

Effect of Choline Deficiency, Carbon Tetrachloride and Ethionine on Body and Liver Weights and Liver Mitochondrial and Microsomal Protein and Total Phospholipid Concentrations of Female Rats

	Treatment[a]					
	Normal female	Choline deficient	Corn oil	Carbon tetrachloride	Saline	Ethionine
Number of Rats	6	6	6	6	6	4
Body weight (g)	183±8[b]	192±7	191±12	195±12	201±7	194±8
Liver weight (g)	7.3±0.5	8.5±0.4	7.5±0.9	7.5±0.1	7.3±0.5	7.1±0.4
Mitochondria						
mg Prot./g liver	46.4±4.4	43.9±4.6	39.1±1.2	39.0±5.8	43.2±3.0	40.6±6.0
μg TPL-p[f]/mg Prot.	6.4±0.6	5.4±1.1[d]	7.5±1.2	7.5±1.1	6.7±0.4	7.3±0.9
Microsomes						
mg Prot./g liver	55.3±5.9	63.1±9.0	41.6±3.7	33.7±5.6[e]	38.7±2.5	35.3±2.8[d]
μg TPL-p/mg Prot.	12.2±1.5	6.0±0.8[e]	15.0±2.4	17.4±2.2[d]	17.1±1.5	17.9±1.8

[a] CCl_4 (0.3 ml in corn oil) was given 7 hr before killing. Ethionine (50 mg in 0.9% saline) was given 12 hr before killing. A choline deficient, low protein diet was fed for seven days prior to killing.

[b] Numbers preceded by ± are standard deviations.

Test of significance was applied to the difference between mean values for the experimental and the control rats for that group. The probability for chance occurrence of this difference was:

[c] $p<0.01$; [d] $p<0.05$; [e] $p<0.005$.

[f] (TPL-p) total phospholipid phosphorus.

Inc., State College, Pa.). The linearity of the detector was verified by quantitating a mixture of fatty acid methyl esters. The fatty acid composition of each phosphatidyl choline fraction is similar to that reported (30).

RESULTS

Table I shows the body and liver weights, protein and total phospholipid levels of liver mitochondria and microsomes following fatty liver induction. An attempt was made to use rats of the same weight for all experiments.

A statistically significant decrease in concentration of microsomal protein (mg/g liver) occurred in the animal administered CCl_4 dissolved in corn oil compared to control receiving corn oil. The administration of ethionine produced a decrease in concentration of microsomal protein compared to control animals receiving saline. The concentration of mitochondrial protein was unaffected. A sta-

TABLE II

Effect of Choline Deficiency, Carbon Tetrachloride and Ethionine on Phosphatidyl Cholines and Phosphatidyl Choline Fractions From Liver Mitochondria and Microsomes of Female Rats[a]

Treatment	Number of rats	Per cent total lecithin-P/total phospholipid-P	Phosphatidyl choline fractions (Per cent total phosphatidyl choline phosphorus)			
			Fraction 1	Fraction 2	Fraction 3	Fraction 4
Mitochondria						
Normal females	6	36.3±2.7[b]	13.7±1.7	7.9±0.8	52.7±3.2	25.7±3.6
Choline deficient	6	27.2±4.0[e]	14.6±3.3	8.0±0.8	35.5±7.0[e]	41.0±2.5[e]
Corn-oil	6	34.9±2.9	9.7±0.6	5.6±1.1	65.9±7.0	19.0±5.5
Carbon tetrachloride	6	38.1±2.7	9.1±1.6	4.7±1.0	60.3±4.0[c]	26.1±2.2[e]
Saline	4	34.2±0.5	9.7±1.4	5.5±1.5	57.7±2.6	27.3±1.3
Ethionine	6	34.6±2.8	7.8±1.2[e]	5.1±1.1	57.9±2.1	29.3±2.2
Microsomes						
Normal females	6	44.9±2.7	7.5±1.4	6.6±2.0	53.3±5.0	32.5±6.9
Choline deficient	6	42.4±4.3	12.4±2.1[e]	7.5±1.0	32.7±6.0[e]	46.0±4.4[e]
Corn oil	6	50.7±6.2	9.7±1.1	4.6±0.6	59.3±7.1	26.3±7.3
Carbon tetrachloride	6	46.9±2.7	9.0±1.1	3.7±0.8[e]	52.2±4.6[c]	35.4±3.9[d]
Saline	4	50.1±1.7	6.9±1.2	4.8±0.5	58.0±3.8	30.7±3.0
Ethionine	6	51.5±2.3	6.9±0.8	4.4±0.6	55.6±5.3	33.2±5.1

[a] *Experimental details* are given in Table I.

[b] Number preceded by ± are standard deviations.

The probability for chance occurrence of difference between mean value for the experimental and the control rats of that group

[c] $p<0.05$; [d] $p<0.01$; [e] $p<0.005$

TABLE III

Fatty Acid Composition of Mitochondrial Phosphatidyl Cholines From Liver of Female Rats Accumulating Fat[a]

	Choline deficient[b]		Carbon tetrachloride[b]		Ethionine[b]	
Fatty acid	Control (6)[d]	Treated (6)	Control (4)	Treated (4)	Control (3)	Treated (5)
14:0[c]	t	0.4±0.2	0.1±0.0	0.1±0.1	0.2±0.1	0.2±0.1
15:0	t	0.2±0.1	0.1±0.0	0.1±0.1	0.1±0.1	0.1±0.0
16:0	13.6±1.8	21.0±2.2	10.4±0.1	11.6±0.6	9.8±0.7	11.3±0.4
16:1	1.0±0.3	1.6±0.2	0.6±0.2	0.8±0.1	0.6±0.2	0.7±0.2
17:0	0.5±0.1	0.3±0.2	0.5±0.1	0.6±0.1	0.3±0.1	0.5±0.1
18:0	30.1±2.4	18.2±1.3	29.7±0.5	29.2±0.8	33.8±1.3	31.1±2.0
18:1	6.9±0.4	12.7±1.4	5.7±0.7	7.6±0.2	5.4±0.5	6.5±0.8
18:2	11.9±1.1	9.4±0.6	6.7±0.9	9.0±0.8	9.4±0.9	11.2±1.1
18:3	.....	0.1	0.3±0.2	0.1	t	0.5±0.2
20:3		0.8±0.5	0.7±0.4	1.4±0.3	1.9±1.1	0.9±0.1
20:4	26.1±1.2	18.9±2.9	33.2±1.3	28.8±1.1	31.0±2.9	30.9±1.3
20:5	t	2.1	0.6	1.4±0.8	t	0.5±0.5
22:5	.....	.....	0.2	.....	.....	.....
22:6	7.5±1.3	12.7±2.2	10.2±0.6	8.4±0.1	7.4±0.9	5.7±1.8
Others	.....	1.5	.....	0.7	0.2	t

[a] Experimental details are given in Table I.

[b] Per cent of total fatty acid by weight.

[c] Number of carbon atoms: Number of double bonds.

[d] Represents the number of determinations.

tistically significant decrease in the total phospholipid phosphorus per milligram of protein of microsomes occurred in the choline deficient animals compared to controls. An increase in microsomal total phospholipid phosphorus per milligram of protein occurred in the animals administered CCl_4 compared to controls receiving corn oil. The concentration of the total phospholipid (μg P/g liver) of mitochondria of the control and choline deficient was 294±39, 232±30 respectively. Similar data were observed for the microsomes of the controls, 677±116 and 353±27 for the choline deficient. If the test (t) of significance was applied to the difference between the controls and choline deficient mean values, the probability (P) for chance occurrence of this difference was $P < .01$. Cornatzer and Walser (32) have reported a decreased concentration of total lecithin in mitochondria of animals fed the similar choline-deficient diet used in the present experiments.

Table II shows the results of the percentage of total lecithin phosphorus per total phospholipid phosphorus and the percentage of the various fractions of phosphatidyl cholines phosphorus per total phosphatidyl choline phosphorus of mitochondria and microsomes. With respect to the phosphatidyl choline fractions, fraction 3 represented 50% of the total phosphatidyl choline in the normal rat in agreement with the findings of Collins (33).

TABLE IV

Fatty Acid Composition of Microsomal Phosphatidyl Cholines From Livers of Female Rats Accumulating Fat[a]

	Choline deficient[b]		Carbon tetrachloride[b]		Ethionine[b]	
Fatty acid	Control (6)	Treated (6)	Control (5)	Treated (6)	Control (4)	Treated (5)
14:0	0.2±0.0	0.2±0.1	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
15:0	0.1±0.0	0.2±0.1	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
16:0	14.7±0.8	21.3±1.9	11.7±0.8	12.5±1.2	11.3±0.6	12.5±0.7
16:1	1.2±0.4	1.7±0.3	0.7±0.1	0.9±0.2	0.9±0.5	1.0±0.3
17:0	0.5±0.1	0.2±0.1	0.5±0.1	0.5±0.1	0.4±0.1	0.5±0.1
18:0	29.9±2.3	18.2±0.7	29.7±2.3	30.4±0.9	34.3±3.0	31.6±1.5
18:1	7.2±1.2	12.4±1.2	6.3±0.5	7.7±0.4	6.2±0.9	7.3±0.7
18:2	14.1±1.2	9.8±0.8	9.6±1.3	10.3±0.8	10.3±1.4	13.1±1.6
18:3	.....	0.1	0.3±0.3	0.6±0.4	0.2±0.3	0.6±0.3
20:3	0.7±0.5	0.8±0.4	0.5±0.3	1.2±0.5	1.0±0.2	0.6±0.4
20:4	25.5±4.2	18.5±2.9	30.5±0.8	26.3±1.2	29.2±2.1	26.2±1.1
20:5	0.9	2.7	1.3	0.5	t	t
22:5	.....	.....	.....	.....	.....	.....
22:6	6.7±1.1	11.9±3.0	7.8±1.3	8.5±0.7	6.3±2.2	6.4±0.7
Other	.....	2.1	0.2	0.5	0.3	0.1

[a] Experimental details are given in Table III.

[b] Per cent of total fatty acid by weight.

TABLE V

Fatty Acid Composition of Mitochondrial Phosphatidyl Choline Fractions From Livers of Female Rats Accumulating Fat

Fatty acid	Control[a]				Choline deficient[a]			
	F-1	F-2	F-3	F-4	F-1	F-2	F-3	F-4
14:0	0.1	0.3	0.2	0.1	0.2	0.4	0.2	0.4
15:0	0.1	0.1	t	0.1	0.1	0.2	0.2	0.2
16:0	17.4	12.9	13.3	16.8	20.5	14.9	16.9	29.2
16:1	t	1.8	0.8	1.0	0.5	1.4	1.2	2.6
17:0	0.6	0.5	0.4	0.5	0.2	0.5	0.9	1.9
18:0	28.3	23.6	33.1	31.5	17.6	19.9	22.8	16.3
18:1	1.4	10.8	4.1	19.7	4.2	9.3	5.8	26.2
18:2	3.5	7.5	9.8	24.7	1.8	5.4	5.7	19.5
18:3	.....	0.3	.....	0.3	0.3	1.5	0.7	0.6
20:3	t	t	0.2	1.6	0.9	4.8	1.2	1.0
20:4	3.8	24.0	38.1	3.4	2.9	12.4	43.4	1.9
20:5	1.0	10.3	.....	.....	1.4	10.9	.....	.....
22:5	.....	6.2	.....	.....	.....	10.2	.....	.....
22:6	0.1	1.8	.....	t	2.3	8.6	1.2	0.3
Others	0.1	1.8	.....	t	2.3	8.6	1.2	0.3

[a] Per cent of total fatty acid by weight.

The major fatty acid in fraction 1 is docosahexaenoic acid. Fraction 4 contains oleic and linoleic acids. Arachidonic acid occurs in fraction 2 and 3. The fatty acids in the various phosphatidyl choline fraction are similar to that reported by Rytter et al. (30). A significant decrease occurred in the percentage of total lecithin per total phospholipid in mitochondria during the production of choline deficiency, similar to that reported by Cornatzer and Walser (32). Ethionine decreased the level of fraction 1 from mitochondria. Corn oil increased the amount of fraction 3 while CCl_4 administered in corn oil retarded or prevented this increase in addition to causing a small change in fraction 2 of liver microsomes. Choline-deficient rats had a marked decrease in fraction 3 from mitochondria and microsomes with an increase in fraction 1 especially in microsomes. A marked increase in fraction 4 occurred in mitochondria and microsomes during the production of choline deficiency. The phosphatidyl choline fractions from choline deficient female rats were similar to those from the normal male rat (34).

Data for the fatty acid composition of phosphatidyl choline after various treatments are presented in Table III and IV. The palmitate-stearate ratio in the mitochondria of control animal was 0.45 and ratio increased to 1.1 in the choline deficient. Similar increase in the ratio was observed in the microsomes. This is in agreement with previous findings for whole liver (16). A decrease in concentration of arachidonic acid occurred in both mitochondria and microsome in the choline defi-

TABLE VI

Fatty Acid Composition of Microsomal Phosphatidyl Choline Fractions From Livers of Female Rats Accumulating Fat

Fatty acid	Control[a]				Choline deficient[a]			
	F-1	F-2	F-3	F-4	F-1	F-2	F-3	F-4
14:0	0.1	0.5	0.2	t	0.2	1.5	0.4	0.6
15:0	0.2	t	0.2	t	0.2	2.1	0.2	0.1
16:0	13.6	10.7	16.9	16.7	18.0	11.5	18.0	26.9
16:1	0.6	1.9	0.6	1.6	0.4	0.3	1.5	2.9
17:0	0.5	0.5	0.4	0.4	0.3	2.1	0.4	0.3
18:0	28.8	28.2	28.1	36.1	19.2	27.3	21.5	18.9
18:1	3.2	8.2	4.4	24.7	2.9	8.6	5.9	26.8
18:2	3.5	6.7	14.5	20.5	1.8	1.9	6.5	18.9
18:3	0.3	0.5	.....	.....	.....	1.9	0.2	0.6
20:3	1.3	t	t	.....	1.7	4.3	1.6	1.3
20:4	3.2	19.7	34.7	.....	2.3	10.6	43.5	2.3
20:5	4.1	12.6	.....	.....	1.5	4.2	.....	.....
22:5	.....	6.8	.....	.....	.....	11.1	.....	.....
22:6	43.4	4.0	.....	.....	51.6	.....	.....	.....
Others	0.9	.....	.....	.....	.....	12.1	t	.....

[a] Per cent of total fatty acid by weight.

TABLE VII

Fatty Acid Composition of Mitochondrial Phosphatidyl Choline Fractions From Liver of Female Rats Accumulating Fat

	Corn oil[a]				Carbon tetrachloride[a]			
Fatty acid	F-1	F-2	F-3	F-4	F-1	F-2	F-3	F-4
14:0	0.1	0.1	0.1	0.1	0.2	0.2	0.2	0.4
15:0	0.2	0.1	0.2	0.1	0.1	0.2	0.1	0.2
16:0	12.8	7.6	10.7	15.3	11.2	6.8	10.6	19.0
16:1	0.3	1.5	0.6	1.1	0.2	1.7	0.6	1.0
17:0	0.5	0.3	2.3	0.8	0.3	0.3	0.5	1.2
18:0	29.1	22.9	33.4	31.9	31.0	22.7	31.8	29.2
18:1	1.7	14.5	2.9	20.8	1.5	11.7	3.7	24.3
18:2	3.9	6.0	6.1	20.7	2.5	5.6	7.2	19.5
18:3	.....	0.3	0.6	0.4	0.2	1.1	0.3	0.4
20:3	.....	0.7	0.4	2.2	t	1.0	0.7	0.6
20:4	4.0	25.2	44.0	4.4	3.6	20.6	44.1	4.0
20:5	t	3.4	.....	.....	1.9	6.7	.....	.....
22:5	.....	9.2	.....	.....	.....	9.6	.....	.....
22:6	47.2	.....	.....	.....	46.0	1.5	.....	.....
Others	0.1	7.5	0.1	0.3	0.9	7.1	0.1	0.2

[a] Per cent of total fatty acid by weight.

cient. Choline deficiency produced an increase in oleic and docosahexaenoic acid in mitochondria and an increase in oleic acid of microsomes. CCl_4 decreased the amount of arachidonic acid found in phosphatidyl choline from mitochondria and microsomes. This is in agreement with previous results for whole liver (17). CCl_4 administration resulted in a decrease in docosahexaenoic acid of mitochondria. Tables V and VI depict the fatty acid composition of phosphatidyl choline fractions from liver mitochondria and microsomes of normal female rats and those on a choline-deficient low protein diet for seven days. The alteration of the palmitate-stearate ratio occurs in fraction 4.

The data in Table VII and VIII give the fatty acid composition of phosphatidyl choline fractions from liver mitochondria and microsomes of control animals receiving corn oil and those administered CCl_4 in corn oil. The fatty acid pattern for the CCl_4 treatment animals are similar to those of the control rats. The concentration of arachidonic in fraction 3 of mitochondria and microsomes of animal administered corn oil (Tables VII and VIII) is greater than those of control animals fed stock diet (Tables V and VI). Docosahexaenoic acid concentration in fraction 1 is greater in mitochondrial and microsomes in animals administered corn oil as compared to those fed stock diet. The data in Table IX and X give the fatty acid composition of phosphatidyl choline fractions from liver mitochondria and microsomes of control animals receiving saline and those administered ethio-

TABLE VIII

Fatty Acid Composition of Microsomal Phosphatidyl Choline Fractions From Livers of Female Rat Accumulating Fat

	Corn oil[a]				Carbon tetrachloride[a]			
Fatty acid	F-1	F-2	F-3	F-4	F-1	F-2	F-3	F-4
14:0	0.1	0.1	0.1	0.2	0.1	0.2	0.2	0.2
15:0	0.1	0.5	0.1	0.2	0.2	0.1	0.1	0.2
16:0	12.3	7.1	10.1	19.2	9.5	7.3	10.7	19.5
16:1	0.2	1.7	0.5	1.2	0.3	1.6	1.0	1.2
17:1	0.5	0.4	0.6	0.6	0.5	0.3	0.5	0.6
18:0	32.0	24.0	34.2	31.0	34.5	32.5	31.9	28.8
18:1	1.8	14.2	3.0	19.0	1.5	9.1	3.1	21.5
18:2	1.8	3.9	7.1	23.3	1.6	3.0	7.8	20.7
18:3	0.2	0.4	0.4	0.4	.....	0.2	.....	0.3
20:3	0.4	3.1	0.2	1.3	0.5	1.0	0.9	1.6
20:4	2.5	22.3	43.2	2.9	1.1	16.7	43.3	4.3
20:5	0.7	7.2	.....	.....	.....	6.0	.....	.....
22:5	.....	9.1	.....	.....	.....	13.8	.....	.....
22:6	46.7	.....	.....	.....	49.9	.....	.....	.....
Others	t	8.2	0.2	0.4	0.1	6.2	0.2	0.4

[a] Per cent of total fatty acid by weight.

TABLE IX

Fatty Acid Composition of Mitochondrial Phosphatidyl Choline Fractions From Liver of Female Rats Accumulating Fat

	Saline[a]				Ethionine[a]			
Fatty acid	F-1	F-2	F-3	F-4	F-1	F-2	F-3	F-4
14:0	0.1	0.2	t	0.2	0.1	0.2	0.2	0.2
15:0	0.1	t	t	t	0.1	0.1	0.1	0.2
16:0	11.3	7.2	8.3	14.8	10.9	6.9	10.4	16.6
16:1	0.1	1.1	0.4	1.1	0.3	1.3	0.8	0.9
17:0	0.4	0.4	0.6	0.6	0.6	0.4	0.5	0.3
18:0	33.8	25.7	35.2	32.3	30.5	23.9	32.4	31.4
18:1	2.0	13.1	2.8	17.4	1.4	12.1	3.3	20.1
18:2	4.0	5.2	4.4	26.5	3.7	6.6	7.5	24.3
18:3	0.1	0.2	0.2	1.1	t	0.5	0.2	0.4
20:3	0.4	3.2	2.0	3.2	1.3	1.8	1.0	1.6
20:4	4.6	21.4	45.9	2.6	4.4	20.0	43.5	3.8
20:5	.....	5.8	.....	.....	0.5	4.1	.....	.....
22:5	.....	10.1	.....	.....	.....	9.6	.....	.....
22:6	43.1	2.5	.....	.....	44.8	5.3	.....	.....
Others	0.2	4.1	0.2	t	1.4	7.7	0.1	0.1

[a]Per cent of total fatty acid by weight.

nine. Arachidonic acid concentration was decreased in fraction 1, 2 and 3 following administration of ethionine, similar to that recently observed by Lyman et al. (35).

DISCUSSION

Artom and Cornatzer (36) have demonstrated that the incorporation of inorganic phosphate ($^{32}P_i$) into liver phospholipids in animals fed a stock diet was 384 c/m compared to 373 c/m in animals fed the 5% casein –5% fat (choline deficient) diet for seven days. Thus it can be concluded that the liver phospholipids synthesis is similar in the animal fed stock diet compared to the animal fed the 5% casein –5% fat diet for seven days to produce a fatty liver. Liver phospholipid synthesis does not decrease in the fatty liver until the histological picture changes to cirrhosis (32,37). The ratio of liver phospholipid P/protein N is unchanged in rats fed the 5% casein –5% fat diet compared to a stock diet, 25% casein –5% fat diet (36).

Glende and Cornatzer (18) have shown that in the animal fed the 5% casein diet for seven days the total liver lipids increased from 37 to 61 mg/g liver. The increase in total liver lipids was due to the increase in neutral lipid fraction (5 to 38 mg/g liver). Maling et al. (19) have shown that CCl_4 produces a three-fold increase in liver triglycerides (11 to 37 mg/g liver). The total liver lipids in the control animals of group 3 (Table I) receiving saline were 8.0 ± 0.8 g/100 g liver and animals receiving DL-ethionine (50 mg in 2 ml of saline) were 10.0 ± 0.5. This in-

TABLE X

Fatty Acid Composition of Microsomal Phosphatidyl Choline Fractions From Livers of Female Rats Accumulating Fat

	Saline[a]				Ethionine[a]			
Fatty acid	F-1	F-2	F-3	F-4	F-1	F-2	F-3	F-4
14:0	0.1	t	0.1	0.2	0.1	0.1	0.1	0.1
15:0	0.2	t	0.2	0.1	0.1	0.1	t	0.1
16:0	10.8	6.7	9.4	19.0	10.0	7.1	10.7	16.9
16:1	0.2	1.4	0.8	1.8	0.1	1.3	0.7	1.1
17:0	0.4	0.6	0.5	0.5	0.5	0.4	0.6	0.5
18:0	32.5	26.1	34.1	31.2	31.1	27.3	32.9	30.5
18:1	1.9	12.2	3.4	17.1	1.4	10.1	3.0	17.9
18:2	1.8	3.4	6.7	27.1	2.1	4.1	9.0	24.2
18:3	.....	0.1	0.4	0.7	0.3	0.6	0.9	1.3
20:3	t	1.3	0.3	1.0	1.8	5.7	2.0	1.7
20:4	3.1	19.7	44.4	1.2	2.9	16.2	40.1	5.3
20:5	t	6.6	.....	.....	2.3	9.0	.....	.....
22:5	.....	14.4	.....	.....	.....	8.0	.....	.....
22:6	49.4	.....	.....	.....	46.5	.....	.....	.....
Others	t	7.4	t	0.2	0.8	9.7	0.1	0.3

[a]Per cent of total fatty acid by weight.

crease of total lipids is similar to that reported by Jensen et al. (20) with Sprague-Dawley female rats receiving DL-ethionine (50 mg in 2 ml of saline).

To assure that the choline deficient animals fed the 5% casein –5% fat diet (Table I) did not have a deficiency of unsaturated fatty acids, the 5% fat diet consisted of 4% safflower oil and 1% cod liver oil. These dietary lipid concentrations are similar to those used by Glende and Cornatzer (18) who studied the liver lipids fatty acid in choline deficiency and concluded that there was no essential difference in the fatty acid patterns of liver lipids from the choline-deficient animals when compared with those receiving a choline supplement. The fatty acid composition of the stock diet, safflower oil and cod liver oil used in the present experiments of Table I has been reported (18).

Excessive liver triglyceride observed in a fatty liver may arise from the diet, from adipose tissue in the form of unesterified fatty acids, and from triglyceride synthesis within the liver. The functions of liver cells are related to organization of cellular organelles, each of which has a characteristic morphology and contains phospholipids. Phosphatidyl cholines are the major lipid components of these cellular membranes (38) and plasma lipoproteins of the cellular organelles (38,39). Numerous studies (33,41,44) have demonstrated that phosphatidyl cholines are metabolically heterogeneous. It has been suggested that choline deficiency may result in an impaired methylation of phosphatidyl ethanolamine to form phosphatidyl-choline (16,45).

Ethionine administration produces a fatty liver in the female rat in the periportal region of the liver (7), inhibits liver protein (8) synthesis, decreases fatty acid oxidation (9), and decreases ATP levels (11, 12). The ethyl groups of ethionine is incorporated into the normal acceptor of the methionine methyl groups (10). Methionine and in most cases ATP can prevent or alleviate the ethionine effects (11, 12). The data presented indicates that ethionine decreased only the concentration of fraction 1 in mitochondria. This fraction contains docosahexaenoic acid. The concentration of arachidonic acid in fraction 1, 2 and 3 was decreased following administration of ethionine.

The concentration of CCl_4 in the liver reaches a maximum within 2 hr, and the amount of triglyceride doubles within 3 hr (46). CCl_4 has been reported to damage microsomes (3,4) followed by alternation of mitochondrial properties (5,6). The arachidonic acid content of liver phospholipid is decreased by CCl_4 (17). Studies reported here indicate a decrease in the level of microsomal protein. Corn oil administration caused an increase in the degree of phosphatidyl choline fatty acid unsaturation, especially in fraction 3. This increase was prevented by administration of CCl_4. The change occurred in mitochondria and microsomes. Since only phosphatidyl choline fraction 3 decreased, the biosynthesis of arachidonic acid or its utilization or the utilization of linoleic acid may be altered by CCl_4 administration. If peroxidation induced by CCl_4 causes changes in lipoproteins (47), it is difficult to justify why arachidonic acid was affected and not other long chain acids unless the other fatty acids are buried within the membrane and not subject to attack.

In addition to causing triglyceride accumulation, choline deficiency results in decreased fatty acid oxidation (15), a decrease in arachidonic acid levels of phospholipid (16), and an altered palmitate-stearate ratio (16). A defect in the methylation of phosphatidyl ethanolamine to form phosphatidyl choline (16,45) has been suggested. Recent work by Rytter et al. (30) demonstrated that phosphatidyl choline fraction 1 is labeled to a greater extent when ^{14}C–ethanolamine was used as the isotope source than when ^{14}C–choline was employed. If impaired methylation of phosphatidyl ethanolamine resulted in a decrease in the amount of fraction 3 because this was the chief source of the phosphatidyl choline, polyunsaturated fatty acids, then fractions 1 and 2 might be expected to decrease but this did not occur. If the availability of arachidonic acid was limiting in choline deficiency or following CCl_4 administration, then the conversion of phosphatidyl ethanolamine to phosphatidyl choline would decrease to conserve phosphatidyl ethanolamine.

The extent of activation of β-hydroxybutyric dehydrogenase has been shown to be related to the degree of phosphatidyl choline fatty acid unsaturation (48). Recently, Kögl et al. (49) obtained data from erythrocytes of different species that suggested a relationship between phospholipid fatty acid composition and membrane permeability. The data presented here show that CCl_4 and a choline deficient-low protein diet do affect the level of phosphatidyl choline fractions differing in the de-

gree of unsaturation of the fatty acids. This alteration could change the properties of the lipoproteins enough to interfere with triglyceride binding or modify cell membranes with a resultant change in triglyceride metabolism or release. This data would further support the recent observation of Lombardi et al. (50) who give evidence of an impaired release of hepatic triglycerides into plasma in choline deficiency. These authors injected intravenously, 1-^{14}C-palmitate and studied the incorporation of the isotope into the triglycerides of liver and plasma of the choline deficient animal.

ACKNOWLEDGMENT

This work was supported in part by a grant from the U.S. Atomic Energy Commission (AT(11-1)-1513) and is part of a thesis submitted by J. E. Miller in partial fulfillment of the requirements for the degree of Doctor of Philosophy. This author was supported by a National Institutes of Health Training Grant Fellowship (2T01-GM 565-6).

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[Received April 4, 1968]

Melting Points of Synthetic Wax Esters

B. T. R. IYENGAR and **H. SCHLENK,** The Hormel Institute,
University of Minnesota, Austin, Minnesota 55912

ABSTRACT

Saturated, monoenoic and dienoic wax esters, $C_{26} - C_{40}$, have been synthesized from even-numbered fatty alcohols and acids. In homologous series of saturated esters, the increments of melting points follow a regular trend except for those esters which have an acid moiety two carbon atoms shorter than the alcohol moiety. These wax esters have melting points higher than interpolation would predict. Monoenoic wax esters with the double bond in the alcohol chain have melting points about 10 C higher than their isomers with the double bond in the acid chain.

INTRODUCTION

ESTERS OF STRAIGHT-CHAIN fatty alcohols with straight-chain fatty acids are often encountered in natural lipids but they have been investigated much less than other non-complex lipids. For example, melting points and crystal structure of fatty acids, methyl esters and glycerides have been studied extensively, but data on wax esters are incomplete and partly in discrepancy. Pure wax esters have been prepared for physical chemical studies and as reference compounds for analyses. We are reporting here on their synthesis and melting points.

EXPERIMENTAL PROCEDURES

Materials

Methyl esters of lauric, arachidic, behenic and oleic acids were purchased from The Hormel Institute Lipid Preparations Laboratory. Pure methyl myristate and stearate were prepared in this laboratory from commercial products by distillation. Methyl palmitate and palmitoleate were prepared from olive oil and mullet body oil, respectively, by distillation of the fatty acid methyl esters and repeated crystallization. All esters used for syntheses were checked by GLC. Their purity was >99% except for methyl palmitoleate which was contaminated with 1.0% C_{15} and 0.8% polyenoic C_{16} esters. The structure of oleate and palmitoleate was checked by ozonolysis.

Fatty alcohols were prepared from the above esters by reduction with $LiAlH_4$ (7). They were recrystallized and their purity was checked by TLC.

Synthesis of Wax Esters

Fatty acid chlorides (8) were reacted with alcohols in the presence of pyridine using diethyl ether as solvent. Procedures are exemplified with the preparation of myristyl palmitate.

A wide test tube equipped with a magnetic stirrer was placed in a silicone bath and connected by a ground glass joint with a condenser carrying a drying tube with $CaCl_2$. Palmitic acid, 1,240 mg (4.85 mmole) in 4 ml benzene, was warmed in the test tube to 65 C. Oxalyl chloride 0.4 ml (4.4 mmole) was added through the condenser and the mixture stirred at 60-65 C for 1 hr. Further 0.2 ml (2.2 mmole) of oxalyl chloride was added at room temperature and the reaction was completed by resuming the above conditions for another hour. Volatiles were then removed by connecting the reaction vessel first to an aspirator and then to an oil pump by a glass tube, 40 × 1 in., packed with KOH pellets. The mixture was stirred to prevent frothing while the pressure was reduced slowly. Finally, vacuum of about 1 mm was applied for 1/2 hr.

The vacuum adapter was replaced by the condenser, and myristyl alcohol, 856 mg (4.0 mmole), dissolved in 8 ml of anhydrous diethyl ether was added to the chloride, followed by anhydrous pyridine, 0.5 ml. After gentle reflux under stirring for 3 hr, the product was transferred with diethyl ether, water and dilute sulfuric acid into a separatory funnel. The ethereal solution of the ester was washed free of pyridine with mineral acid. After further washing with aqueous alkali and water, and drying over anhydrous sodium sulfate, the solvent was evaporated leaving a residue of 2 g of crude myristyl palmitate. TLC revealed traces of contaminants which were removed by column chromatography.

A column, 32 × 2.3 cm, was packed with 55 g of activated silicic acid (Mallinckrodt, 100 mesh), slurried in Skellysolve B which contained 3% diethyl ether. Myristyl palmitate, 2 g, was chromatographed with the same solvent mixture. Pure ester was eluted in the volume from 100 to 260 ml and was followed by a smaller amount of slightly contaminated ester. The pure fraction was crystallized from

TABLE I
Melting Points of Saturated Wax Esters

Ester	Abbreviated alc-acid	M.P. in C
Myristyl laurate	14-12	40.0-40.4
Palmityl laurate	16-12	41.2-41.6
Myristyl myristate	14-14	44.4-45.4
Palmityl myristate	16-14	51.7-52.0
Stearyl myristate	18-14	52.0-52.2
Arachidyl myristate	20-14	54.5-54.9
Myristyl palmitate	14-16	49.2-49.7
Palmityl palmitate	16-16	53.6-54.0
Stearyl palmitate	18-16	59.6-59.9
Arachidyl palmitate	20-16	60.6-60.9
Myristyl stearate	14-18	52.5-52.8
Palmityl stearate	16-18	58.5-59.0
Stearyl stearate	18-18	61.6-61.9
Arachidyl stearate	20-18	67.4-67.8
Behenyl stearate	22-18	67.4-67.6
Myristyl arachidate	14-20	54.8-55.2
Palmityl arachidate	16-20	61.1-61.3
Stearyl arachidate	18-20	65.8-66.3
Arachidyl arachidate	20-20	68.9-69.2

diethyl ether and recrystallization did not change the melting point. The yield was 1.5 g pure myristyl palmitate (83% in reference to myristyl alcohol).

Other wax esters were synthesized in the same manner with only minor modifications. Saturated wax esters C_{38} and C_{40} are rather insoluble and were applied to the columns by impregnating silicic acid with them and then transferring this mixture to the top layer of the adsorbent. The upper part of these columns was heated to about 40 C. The outflow drip

TABLE II
Melting Points of Unsaturated Wax Esters[a]

Ester	Abbreviated alc-acid	M.P. in C
Monounsaturated		
Myristyl palmitoleate	14:0-16:1	2.5- 3.0
Palmityl palmitoleate	16:0-16:1	10.5-11.0
Stearyl palmitoleate	18:0-16:1	17.8-18.3
Arachidyl palmitoleate	20:0-16:1	28.5-28.8
Myristyl oleate	14:0-18:1	7.9- 8.5
Palmityl oleate	16:0-18:1	16.5-17.0
Stearyl oleate	18:0-18:1	23.8-24.2
Arachidyl oleate	20:0-18:1	31.5
Palmitoleyl myristate	16:1-14:0	13.8-14.3
Palmitoleyl palmitate	16:1-16:0	22.3-22.6
Palmitoleyl stearate	16:1-18:0	26.8-27.2
Palmitoleyl arachidate	16:1-20:0	35.0-35.5
Oleyl myristate	18:1-14:0	19.7-20.4
Oleyl palmitate	18:1-16:0	28.0
Oleyl stearate	18:1-18:0	34.0-34.5
Oleyl arachidate	18:1-20:0	39.5-40.0
Diunsaturated		
Palmitoleyl palmitoleate	16:1-16:1	-18.0 to -17.0
Oleyl palmitoleate	18:1-16:1	-12.0 to -11.0
Palmitoleyl oleate	16:1-18:1	-11.0 to -10.0
Oleyl oleate	18:1-18:1	- 4.0 to - 3.5

[a] Double bonds are in position 9.

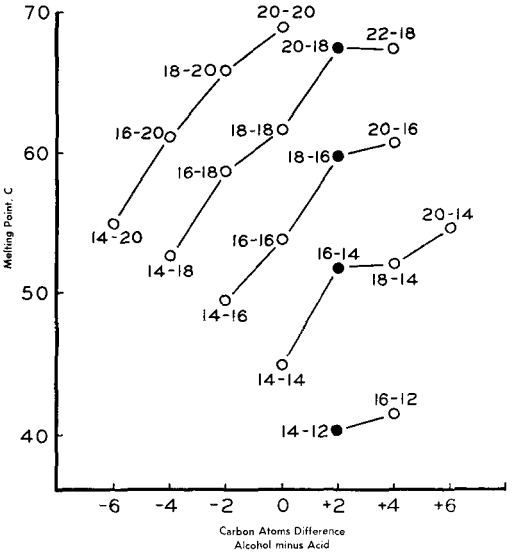

FIG. 1. Melting points of saturated wax esters. Melting points higher than interpolation would predict are marked by filled circles.

tip and the siphon to the fraction collector were washed periodically with chloroform to remove any crystals that may have formed. Precautions against autoxidation were taken in preparing unsaturated wax esters. Some of the purified unsaturated wax esters crystallized from Skellysolve B or from diethyl ether only at low temperature, –30 to –65 C.

The purity of the wax esters was checked by TLC and GLC. To test the sensitivity of these methods, fatty alcohol, acid and methyl ester, 1% of each, were added to one of the preparations. The mixture was chromatographed on Silica Gel G, which had been activated at 120 C for 4 hr. Skellysolve B—diethyl ether—glacial acetic acid (85:15:1) was the solvent. All components were easily detected. Similarly, it was ascertained that GLC would detect 1% of a contaminating homologous wax ester. GLC conditions were as previously described (9-11). In reference to these tests the purity of the wax esters was >99%, except esters containing palmitoleyl chains where purities were >98%.

Melting Points

The melting points of all saturated wax esters were determined on a Kofler Micro Hot Stage (A. H. Thomas Co.) by raising the temperature at a rate of 1-2 C/min. The thermometer was corrected according to melting points of standard substances (A. H.

Thomas Co.). Melting points of unsaturated wax esters were usually taken in small tubes which were attached to a thermometer and cooled slowly in a stirred water bath. Small pieces of ice were added until the substance solidified completely. The bath was kept at that temperature for 1-2 hr and was then allowed to warm up gradually. Acetone and dry ice were used as coolant with diunsaturated wax esters.

DISCUSSION

Even-numbered saturated and monounsaturated components, C_{12}–C_{20} have been used for the syntheses. Accordingly, the bis-homologous series of wax esters, $C_{26} - C_{40}$, contains saturated, monounsaturated and diunsaturated esters, including numerous isomers. In the following, the common abbreviated nomenclature is used for alcohol and acid moieties, listing the former first: 14:0-16:0 stands for myristyl palmitate, 16:1-18:1 for palmitoleyl oleate, and so on.

Melting points of the synthetic wax esters are listed in Tables I and II. Melting points for some of these compounds have been reported in the literature and have been quoted in reviews (1-3) as well as in more current publications (4,5). Discrepancies among literature data or between them and our data probably are due to impurities which were not recognized at the time when analytical methods were less sensitive than now. The purity of our preparations is, according to GLC and TLC, better than, or close to 99%. The esters were crystallized from one or more of the solvents, diethyl ether, Skellysolve B (essentially n-hexane, bp 60-70 C) and acetone and the melting points remained unchanged within ±0.3 C. Polymorphism, as it has been reported from some x-ray diffraction studies (4,6), was not indicated by melting points.

Increments of melting points in the saturated series are approximately 5 C for homologs of the shorter chain lengths. They decrease to about 3 C for the longer chain homologs. There is, however, one exception which appears in each of the even-numbered homologous series. It is seen in Fig. 1 that pairs such as 14:0-12:0 and 16:0-12:0, 16:0-14:0 and 18:0-14:0, and others, have melting points equal or much closer than other pairs of the respective homologous series. Expressed in general terms, the melting points of saturated esters, Alc_{C_n}-$Acid_{C_{n-2}}$, are higher than predicted by interpolation from melting points of other members of that homologous series. Such a rule had not become apparent from earlier investigations. The exceptions are consistent in several series, and this makes it unlikely that contaminants are the cause.

Examination of the data in Table I shows that among saturated wax esters, isomers having equal or close to equal chain lengths of alcohol and acid moieties have the highest melting points. This is, of course, not valid for extreme differences of chain length. For example, methyl hentriacontanoate with a total of 32 carbon atoms and its isomer, ethyl triacontanoate, melt at 74.5 C and 69 C, respectively (3). Both melting points are higher than those of typical wax esters having 32 carbon atoms.

Monoenoic wax esters (Table II) which are unsaturated in the alcohol moiety have higher melting points than their isomers with the double bond in the acid moiety. It was observed that regardless of solvent, monoenoic esters of the former type crystallize in heavy thick clusters, whereas the latter esters crystallize in flaky leaflets similar to those of saturated wax esters.

ACKNOWLEDGMENTS

This work was supported by U. S. Public Health Grants HE 05363 and HE 08214, and by The Hormel Foundation.

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[Received May 8, 1968]

Fatty Acid Composition of Adult *Schistosoma mansoni*

THOMAS M. SMITH, THOMAS J. BROOKS, JR. and **HAROLD B. WHITE, JR.,**
Departments of Biochemistry and Preventive Medicine,
The University of Mississippi School of Medicine, Jackson, Mississippi 39216

ABSTRACT

The fatty acid composition of triglyceride and total phospholipid fractions of adult *Schistosoma mansoni* has been examined. Both triglyceride and phospholipid contained fatty acids varying in chain length from 12 through 24 carbons; trace amounts of shorter chain components were found in the triglyceride fraction. A docosahexaenoic acid in the triglyceride fraction represented the highest degree of unsaturation encountered. Branched chain fatty acids of 16 and 18 carbons were found in both phospholipid and triglyceride. Examination of fatty acids from fluke total lipid revealed the presence of small amounts of odd numbered carbon fatty acids varying in chain length from 13 through 23 carbons.

INTRODUCTION

SCHISTOSOMIASIS IS A PARASITIC disease of considerable medical and economic importance. Throughout the world nearly 200 million people are currently infected with one of the three species of blood flukes (1); the disease is particularly prevalent in the Orient (2). In the Western Hemisphere, schistosomiasis mansoni is widely distributed in Brazil, certain of the Caribbean Islands and in some areas of Puerto Rico (3). The accumulation of eggs from this blood fluke in the human liver and gut produces extensive fibrosis. Prognosis is poor in chronic cases with advanced cirrhosis of the liver (3).

Carbohydrate and protein metabolism of *Schistosoma mansoni* have been investigated (4, 5) but the lipid composition of this important blood fluke has received little attention (6). The purpose of this paper is to identify the fatty acids found in the triglyceride and phospholipid fractions of *S. mansoni* total lipid as an aid to the future understanding of lipid metabolism which may stimulate the development of new chemotherapeutic, prophylactic or suppressive agents.

MATERIALS AND METHODS

White Swiss mice or DBA/2 mice were maintained on a diet of Purina rat chow (Ralston Purina Company, St. Louis, Mo.) and water ad lib. Mice infected for 6 to 10 weeks with the Puerto Rican strain of *S. mansoni* were killed by skull fracture and the mature flukes dissected from the mesenteric venules. Prior to extraction with chloroform–methanol (2:1, v/v) (7), these trematode parasites were washed in three changes of physiological saline and counted.

Tissue lipid was separated into classes by ascending TLC on 20 x 20 cm glass plates coated with a 250 μ silica gel G adsorbent layer (E. Merck AG, Darmstadt, Germany). Normal hexane–diethyl ether–acetic acid (70:30:1, v/v/v) was used as the developing solvent (8). Reference mixtures for lipid class separations by TLC and methyl ester standards were obtained from the Hormel Institute, Austin, Minn.

Prior to the application of lipid samples all chromatoplates except those coated with a 250 μ layer of silica gel HR (E. Merck AG, Darmstadt, Germany) were washed with methanol–diethyl ether (80:20, v/v) to remove impurities in the silica gel layer (9). All solvents were routinely examined for contaminants by dissolving any residue remaining after evaporation of 100 ml in 10 μl of *n*-hexane and injecting this solution into a gas chromatograph. Operating conditions were always those used in subsequent analyses of fluke lipid. Contaminated solvents were purified by appropriate procedures detailed elsewhere(10). When necessary, 2,7–dichlorofluorescein and UV light were used to visualize standard class separations.

Silica gel bands containing the separated components were scraped into individual 5 ml round bottom flasks. About 2 ml of 6% sulfuric acid in anhydrous methanol was added to lipid class or total lipid samples and each mixture refluxed for 6 hr at 80° C.

Methyl esters were routinely extracted into hexane and separated from other extractable reaction products by TLC using an *n*–hexane–diethyl ether–acetic acid (90:10:1, v/v/v) developing solvent (11). *S. mansoni* phospholipid components were further studied by rechromatographing the phospholipid fraction from above in chloroform–methanol–water (65:25:4, v/v/v) and spraying the developed

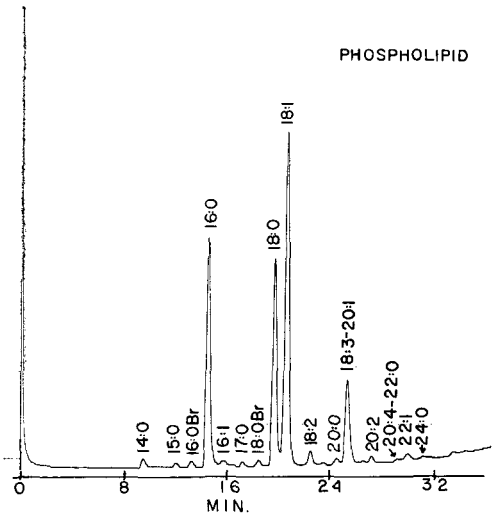

FIG. 1. GLC recording of fatty esters from adult *S. mansoni* phospholipid from 470 pair. Glass column 4 ft × 4 mm i.d., packed with 5% DEGS on Diatoport S.

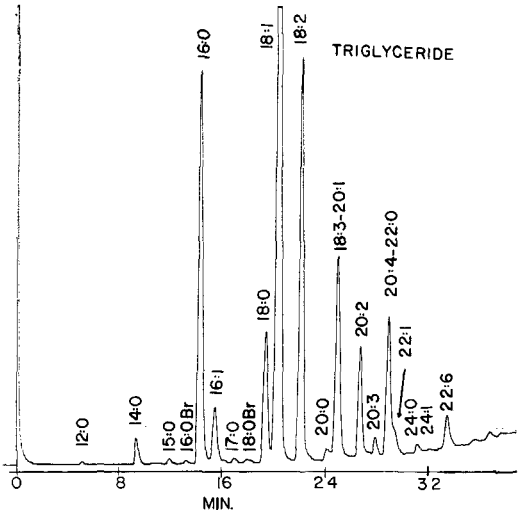

FIG. 2. GLC recording of fatty esters from adult *S. mansoni* triglyceride from 470 pair. Column specifications detailed in Fig. 1 legend.

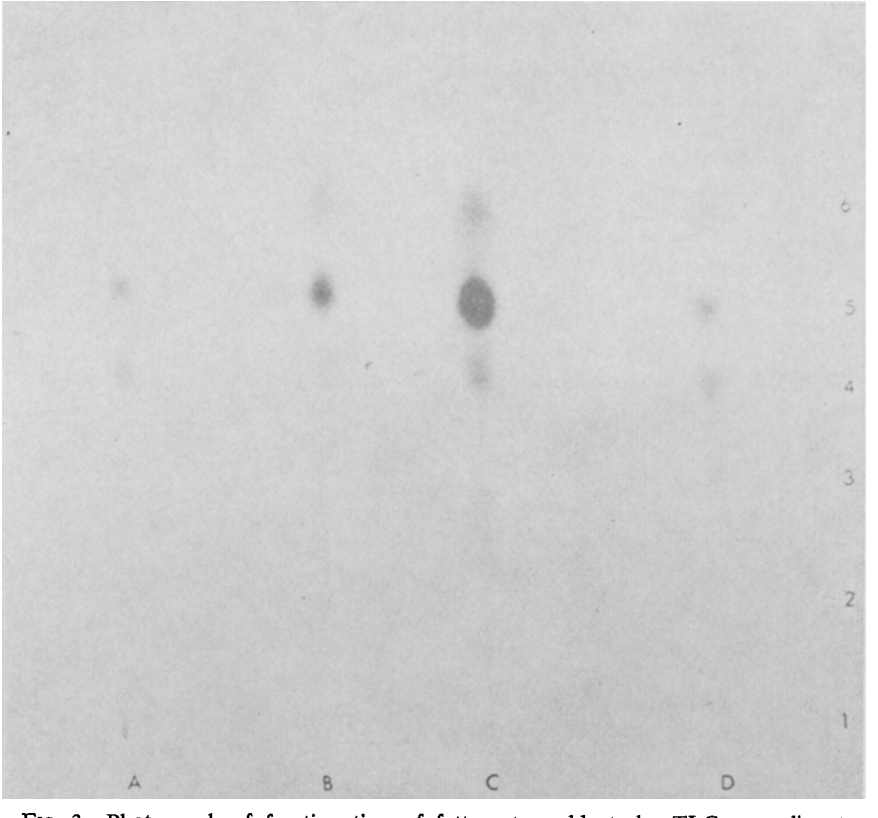

FIG. 3. Photograph of fractionation of fatty ester adducts by TLC according to number of double bonds. A, D, 1-6, standard mixture, in progressive order, from the origin: 1) docosahexaenoate and eicosapentaenoate, 2) arachidonate, 3) linolenate, 4) linoleate, 5) oleate, 6) stearate. B, C, adducts of fatty esters from adult *S. mansoni* total lipid, 1-6, in progressive order from the origin: 1) hexaenes and pentaenes, 2) tetraenes, 3) trienes, 4) dienes, 5) monoenes, 6) saturates.

plates with either ammonium molybdate–perchloric acid reagent or ninhydrin and comparing calculated R_f values with recorded values for phosphatides (12).

Fatty acid methyl esters were separated and qualitatively identified by two GLC procedures. In the first, methyl esters from fluke total lipid or lipid classes were chromatographed on either an F and M model 400 or 402 gas chromatograph equipped with hydrogen–flame ionization detectors. Results are shown in Figs. 1 and 2. The column used in both instruments was a 4 ft × 4 mm i.d. glass tube packed with 5% polydiethyleneglycolsuccinate (DEGS) coated on 80–100 mesh Diatoport S. Operating conditions were helium flow rate 75 ml/min, temperature programming from 100–210 C at 3°/min and 400 attenuation of signal. Identification in this instance was made by comparison of unknown peak relative elution temperatures with those of standard methyl esters as previously described (8,13). In the two column technique used as the second GLC procedure, the problem of overlapping components so evident in Figs. 1 and 2 was resolved by collecting chain length fractions from the effluent port of either an F and M model 400 gas chromatograph as described elsewhere (14) or from the effluent port of an F and M model 500 gas chromatograph. In the latter chain length collection process, an 8 ft. × ¼ in. i.d. stainless steel column packed with 20% S. E. 30 on 60–80 mesh Chromosorb W was used. Operating conditions were helium flow rate 120 ml/min, temperature programming from 250–375C at 4°/min, and a signal attenuation of 1. Block temperature was 390C and the effluent port was wrapped with asbestos string to a width of 2 in. to prevent condensation of higher molecular weight methyl esters in the effluent tube during the collection process. All collected chain length fractions were analyzed for double bond content by GLC on 5% DEGS with either an F and M model 400 or model 402 instrument as described above.

To further aid in establishing degrees of unsaturation in individual fatty acids from fluke total lipid, phospholipid, or triglyceride, a complementary TLC, GLC procedure (15) modified by initially separating the methoxy, bromomercuri–adducts on longer (20 × 40 cm) silica gel HR coated chromatoplates was used (Fig. 3). After adduct decomposition, the component chain lengths of the saturated, monoenoic and dienoic fatty acids were determined by GLC of recovered methyl esters on 5% DEGS using operating conditions detailed above.

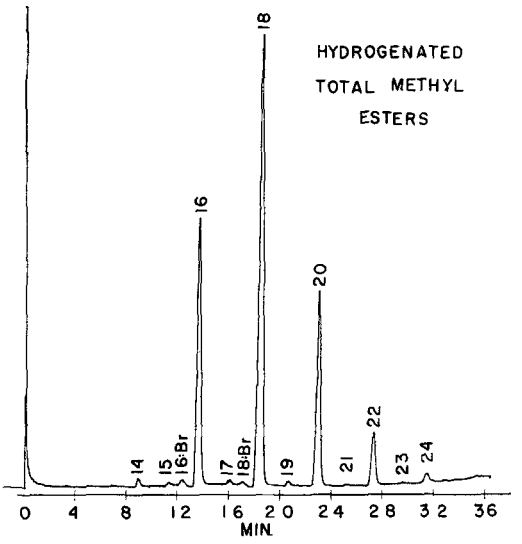

FIG. 4. GLC recording of hydrogenated fatty esters from adult *S. mansoni* total lipid. Column specifications detailed in Fig. 1 legend.

Hydrogenated methyl esters from fluke total lipid, phospholipid and triglyceride (16) were analyzed by GLC on 5% DEGS to assist in resolving the complex general composition of each mixture, to verify chain length composition and to help in detecting the presence of branched chain fatty acids (Fig. 4).

Peak areas were determined by multiplication of peak height by width at half-height. Percentages listed in Tables I and II represent the mean of the percentages of the total area under the curves contained in the peaks.

RESULTS AND DISCUSSION

The first larval stages of all digenetic trematodes develop in molluscan intermediate hosts. This fact distinguishes this group of parasitic flatworms from all others. These original molluscan parasites apparently later developed an association with vertebrate hosts (17).

Initial larval stages of *S. mansoni* develop in fresh water snails and this fresh water origin of these blood flukes is reflected in the large amounts of C_{16} and C_{18} acids (18) found by us in adult *S. mansoni* triglyceride, phospholipid and total lipid (Table I). Indeed, the predominance of C_{16} and C_{18} fatty acids in man, monkeys and wild rodents may be par-

TABLE I

Fatty Acid Composition of Adult *Schistosoma mansoni* Tissue Lipids

Fatty Acid	Total Lipid[a]				Triglyceride[b]				Phospholipid[c]			
	Mean	S.D. Area %	Min.	Max.	Mean	S.D. Area %	Min.	Max.	Mean	S.D. Area %	Min.	Max.
12:0	0.1	0.1	0.1	0.2	0.2	0.1	0.1	0.2	Trace[d]			
13:0[e]	Trace				Trace				Trace			
14:0	0.7	0.5	0.5	1.2	0.8	0.4	0.4	1.2	0.7	0.3	0.4	1.2
15:0	0.3	0.1	0.2	0.4	Trace		Trace	0.1	0.4	0.1	0.2	0.5
16:0Br	Trace				0.2		Trace	0.2	1.2	0.3	0.7	1.6
16:0[i]	17.6	1.4	16.3	19.5	14.5	6.0	7.9	19.5	28.6	5.1	22.6	36.1
16:1	1.1	0.0	1.0	1.1	2.2	0.9	1.1	2.9	0.6	0.1	0.5	0.9
17:0	0.5		0.5	0.5	0.4		Trace	0.4	0.4	0.1	0.3	0.4
18:0Br	0.3		Trace	0.3	0.2	0.2	0.1	0.4	0.6	0.1	0.5	0.8
18:0[i]	11.8	0.3	10.7	13.2	5.3	1.4	3.6	6.3	21.8	2.8	19.0	27.4
18:1[i]	22.2	1.5	21.0	24.8	32.4	1.0	31.2	33.9	17.2	6.1	12.8	34.2
18:2[i]	9.4	0.3	9.1	9.8	14.7	1.1	13.4	15.8	2.7	1.5	1.4	5.1
18:3	Trace				Trace				Trace			
19:0	0.6		0.4	0.8	0.4	0.1	0.4	0.5	0.4	0.1	0.4	0.5
20:0	0.6	0.1	0.4	0.7	0.4	0.1	0.3	0.5	0.8	0.2	0.5	1.2
20:1[i]	10.7	0.8	9.9	11.9	8.8	3.0	6.6	12.2	11.6	1.5	9.4	14.0
20:2	4.3	0.4	3.7	4.7	3.1[f]		3.1	3.2	2.1	1.2	0.8	4.0
20:3					1.1[f]		1.0	1.1				
20:Unsat. Unid.									0.6[g]		0.5	0.6
20:4	9.5[h]	0.4	9.4	10.0	5.7[f]		5.6	5.9	Trace		Trace	0.4
20:5	0.4	0.1	0.3	0.6	0.4[f]	0.1	0.3	0.5				
21:0	Trace				Trace				Trace			
22:0	0.9	0.1	0.8	1.0	1.3[f]	1.1	0.9	1.8	0.4	0.1	0.3	0.5
22:1					0.9[f]		0.8	1.1	4.0	3.0	1.3	8.4
22:Unsat. Unid.									2.1[g]		2.1	2.2
22:2					0.2[f]		0.2	0.2				
22:3	0.8	0.4	0.3	1.2	Trace							
22:4					1.9[f]		1.7	2.2				
22:5					0.3[f]		0.3	0.4				
22:6	1.6	0.9	0.7	2.5	1.0[f]		0.9	1.2				
23:0	Trace				Trace				Trace			
24:0[j]	5.0	0.5	4.3	5.5	0.4	0.2	0.3	0.5	0.6	0.2	0.4	0.8
24:1[j]	0.2	0.1	0.2	0.3	2.9	1.1	2.0	4.3	3.2	3.2	0.6	7.6
Unid.	1.4	1.2	0.1	2.5	0.3	0.1	0.3	0.4				

[a] Six determinations on two samples (145 pair and 205 pair + 2 male flukes).
[b] Five determinations on two samples (243 pair and 200 pair).
[c] Nine determinations on four samples (243 pair, 1088 pair, 1087 pair + 180 males and 360 pair + 28 males).
[d] Trace = less than 0.1% of total area.
[e] Odd chain length data from hydrogenated samples.
[f] Three determinations on one sample (243 pair).
[g] Found in three determinations on 360 pair + 28 males only.
[h] 20:4 and 22:1 in total lipid.
[i] Major fatty acids composing 10% or more of total or either fraction. Unid. = unidentified. Unsat. = unsaturated fatty acid. Br—branched chain fatty acid.
[j] May represent a mixture with 22:3 or 22:4.

tially responsible for their selection as definitive hosts. An examination of *S. mansoni* cercarial total lipid fatty acid composition revealed a similar prevalence of C_{16} and C_{18} fatty acids in the larval stage which penetrates the skin to establish the parasite in the mesenteric venules and liver of primates and rodents (8).

The fluke phospholipid fraction was found to contain materials corresponding in R_f to amino acids, lysolecithin, sphingomyelin and cardiolipin. Results of analyses of fatty acid moieties from *S. mansoni* total lipid, triglyceride and phospholipid are shown in Tables I and II. The triglyceride fraction contained 31 fatty acids varying in chain length from 12 through 24 carbons. Occasionally traces of shorter chain fatty acids were seen in GLC strip chart recordings. A similar chain length composition was found among the 27 fatty acids from *S. mansoni* total lipid and the 26 fatty acids of the phospholipid fraction (Tables I and II). A fatty acid composition nearly as complex has been reported for neutral lipid from female *Moniliformis dubius*, a thorny headed acanthocephalan parasite of rats which contained some 29 fatty acids differing in chain length from 10 through 22 carbons (19). A study of neutral lipid from the female swine acanthocephalan *Macracanthorhyncus hirudenaceus* revealed 16 fatty acids varying in chain length from 10 through 20 carbons (19). Analyses of major fatty

TABLE II

Hydrogenated Fatty Acid Methyl Esters from Adult *Schistosoma mansoni* Tissue Lipids

Fatty Acid	Total Lipid			Triglyceride[d]				Phospholipide			
	Swiss Mice		DBA Mice[f]	Swiss Mice				Swiss Mice			
	(1)[a]	(2)[b]	(1)[c]	Mean	S.D.	Min.	Max.	Mean	S.D.	Min.	Max.
	Area %			Area %				Area %			
Less than 14:0	Trace[g]	Trace	1.0	Trace				Trace			
14:0	1.0	0.5	0.4	0.8	0.2	0.7	1.0	0.4	0.1	0.4	0.5
15:0	0.5	0.3	Trace	Trace				0.3	0.1	0.2	0.4
16:0Br	0.9	Trace		Trace				Trace			
16:0[h]	24.7	28.6	19.8	17.0	0.2	16.8	17.2	21.6	0.6	21.1	21.4
17:0	0.5	0.5	0.7	0.4		Trace	0.4	0.4	0.1	0.3	0.4
18:0Br	0.6	Trace		Trace				Trace			
18:0[h]	46.8	44.6	40.1	54.2	0.6	53.4	54.7	37.4	0.9	36.6	38.3
Unid.		0.5	0.9					0.8	0.1	0.7	0.9
19:0	0.4	0.8	0.5	0.4	0.1	0.4	0.5	0.4	0.1	0.4	0.5
20:0[h]	18.0	18.9	20.1	21.6	0.8	20.8	21.7	23.4	0.2	23.2	23.7
21:0	Trace	Trace	Trace	Trace				Trace			
22:0	5.5	3.8	7.0	4.5	0.1	4.1	5.0	13.3	0.2	12.6	13.9
23:0	Trace	Trace	Trace	Trace				Trace			
Unid.			0.5								
24:0	1.1	1.5	9.0	1.1	0.1	1.1	1.3	2.0	0.1	1.9	2.0

[a] One determination on total lipid from 330 pair.
[b] One determination on total lipid from 863 pair.
[c] One determination on total lipid from 229 pair + 26 males.
[d] Four determinations on triglyceride from 722 pair + 13 males.
[e] Four determinations on phospholipid from 328 pair.
[f] Dilute Brown Agouti mice.
[g] Trace = less than 0.1% of total area.
[h] Major fatty acids composing 10% or more of the total or either fraction. Unid. = unidentified. Br = branched chain fatty acid.

acids from *Spirometra mansonoides* sparganum larval lipid resulted in the detection of 15 acids varying in chain length from 14 through 22 carbons in the total lipid (20). The 14 fatty acids detected in the sparganum neutral lipid and the 15 fatty acids from its phospholipid exhibited a similar chain length constitution. In adult *S. mansonoides,* a tapeworm from domestic cats, 16 fatty acids varying in chain length from 14 through 22 carbons were identified in both neutral lipid and phospholipid. As in adult *S. mansoni,* the C_{16} and C_{18} acids were found to be generally predominant in lipid from each parasite mentioned above, however, the C_{24} acids detected in adult *S. mansoni* represent the longest chain length detected in lipids from any of these parasites (19,20).

A comparison of adult *S. mansoni* triglyceride and phospholipid fatty acid components with the same fractions from normal uninfected white Swiss mouse blood revealed a more complex fluke triglyceride fatty acid constitution but a very similar fatty acid composition for fluke and mouse blood phospholipid except that the predominant 20 carbon unsaturated fatty acid in fluke phospholipid was 20:1 in contrast to 20:4 in mouse blood phospholipid (21,22). *S. mansoni* thus appears to be capable of synthesizing its own triglyceride. It has been reported that *S. mansonoides* is able to synthesize triglyceride, sterol ester and phospholipid from exogenously supplied fatty acids and sterols (20).

Branched chain fatty acids of 16 and 18 carbons were found in fluke total lipid, phospholipid and triglyceride along with small amounts of odd numbered carbon fatty acids differing in chain length from 13 through 23 carbons (Tables I and II). Trace amounts of 13 carbon branched chain fatty acids were identified in neutral lipid from *M. hirudenaceus* while *M. dubius* neutral lipid contained traces of a 14 carbon branched chain acid. Small quantities of odd numbered carbon fatty acids differing in chain length from 11 through 17 carbons in *M. dubius* and 13 through 17 carbons in *M. hirudenaceus* have been reported (19). Neither branched chain nor odd numbered carbon chain acids were detected in lipid from the larvae or adult stage of *S. mansonoides* (20).

The presence of highly unsaturated eicosatetraenoic acids in *S. mansoni* phospholipid and triglyceride and eicosapentaenoic and docosahexaenoic acids in the fluke triglyceride frac-

tion at least suggests that pathways for fatty acid interconversions could exist in adult *S. mansoni*.

ACKNOWLEDGMENTS

This investigation was supported in part by USPHS Grant AI 07546, National Institute of Allergy and Infectious Diseases. H. B. White is a research career development awardee (6-K3-HE-18,345), United States Public Health Service. Technical assistance was given by Mrs. Katye R. Summerlin and Mrs. Shirley S. Powell.

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[Received May 17, 1968]

Two Synthetic Phosphonate Analogs of Lecithin

ARTHUR F. ROSENTHAL and **MANUEL POUSADA**, Department of Laboratories, The Long Island Jewish Hospital, New Hyde Park, New York 11040

ABSTRACT

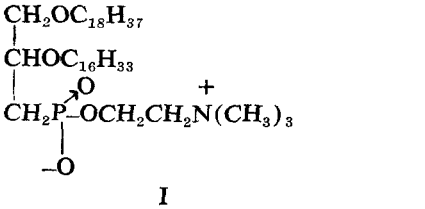
The diether-phosphonate lecithin analog DL-2-hexadecoxy-3-octadecoxypropylphosphonylcholine was prepared in 55% overall yield by reaction of 2-hexadecoxy-3-octadecoxypropylphosphonic acid with choline iodide and *p*-toluenesulfonyl chloride, and purified chromatographically.

The diester-phosphonate lecithin analog 1,2-dipalmitoyl-*sn*-glycero-3-[2'-trimethylammonium) ethylphosphonate] was prepared from *sn*-glycerol-1,2-dipalmitate and 2-(trimethylammonium) ethylphosphonyl dichloride, and the reaction mixture purified chromatographically. The lecithin analog was obtained in an anhydrous form.

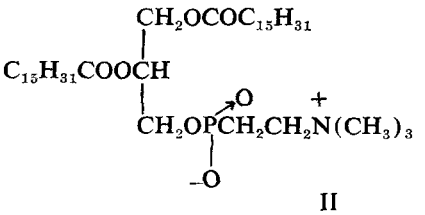
INTRODUCTION

DURING THE PAST FEW YEARS the synthesis of phosphatide analogs of several new types, containing as a common feature the replacement of a P-O-C moiety by the non-hydrolyzable C-P group, has been reported (1-12). This interest in what until recently would have been considered the biologically exotic phosphonate group in lipids appears to have been stimulated from two directions: the finding of phosphonate-containing lipids in nature (13-16), and the aim of synthesizing biologically stable inhibitors of phosphatide-degrading enzymes (17,18).

Syntheses of two phosphonate-containing lecithin analogs (I and II) are described below.



I



II

The first of these, DL-2-hexadecoxy-3-octadecoxypropylphosphonylcholine (I), was prepared from the corresponding phosphonic acid (8) by condensation with choline iodide in the presence of *p*-toluenesulfonyl chloride. Purification of the product was accomplished in its free acid-trifluoroacetate form by chromatography on a silicic acid-celite column. It was possible to use trichloroacetonitrile as the condensing agent, but a very dark product was obtained which was difficult to free from traces of pigment even chromatographically.

The product was obtained as a white powder considerably more soluble in chloroform and other organic solvents, and more dispersible in water, than the corresponding phosphonate-cephalin (4). The lecithin analog was analyzed correctly for a monohydrate form. It was found to be a powerful inhibitor of the phospholipase C reaction (19).

1,2-Dipalmitoyl-*sn*-glycero-3-[2'-(trimethylammonium)ethylphosphonate] (II), has previously been synthesized by Baer and Stanacev (7), who also prepared the corresponding dimyristoyl and distearoyl homologs. These authors reacted 1,2-diacyl-*sn*-glycerols with the monoacid chloride of 2-bromoethylphosphonic acid, and treated the products with trimethylamine to give the phosphonate lecithins.

It seemed of interest to investigate whether the same compounds could be formed by direct reaction of the 1,2-diacyl-*sn*-glycerol with the acid chloride of the zwitterionic 2-(trimethylammonium)-ethylphosphonic acid (20). Accordingly, the acid chloride (presumably dichloride) was prepared from the zwitterion by treatment with phosphorus pentachloride and reacted in the presence of pyridine with *sn*-glycerol-1,2-dipalmitate. The crude reaction product on chromatographic separation yielded among a number of substances a compound apparently identical to that prepared by Baer and Stanacev (7) on the basis of its melting point and infra-red spectrum.[1] A second product,

[1]The specific rotation found for this compound (+9.6°) is considerably higher than that reported by Baer (+7.5°). The reason for this discrepancy is not definitely known; however, our rotation was obtained in much more dilute solution than Baer's, in a different solvent [chloroform vs. chloroform-methanol (3:1)], and on a photoelectric instrument (Bendix Automatic polarimeter; Bendix Corp., Cincinnati, Ohio) rather than an optical polarimeter.

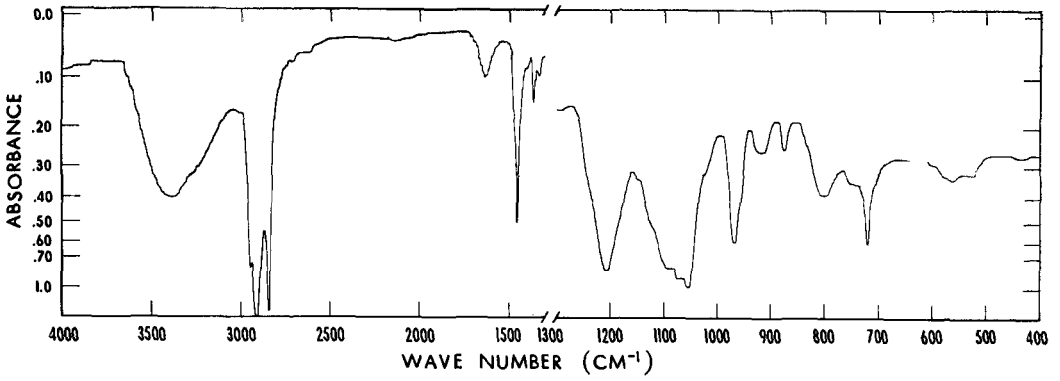


FIG. 1. Infrared spectrum of DL-2-hexadecoxy-3-octadecoxypropylphosphonylcholine.

eluted from this column just before the lecithin analog, had properties very similar to the latter, including the same elemental analysis. The structure of this by-product is not known at present; it is evidently not an *sn*-1,3-diacyllecithin analog, since it was optically active, nor a trimethylammonium salt of a diglyceride vinylphosphonate (potentially formed via an elimination reaction), since treatment with trichloroacetic and trifluoroacetic acids, as well as Amberlite IR-120(H^+), failed to remove any nitrogen. The yield of the by-product is sharply increased if thionyl chloride, rather than phosphorus pentachloride, is used to form the acid chloride.

The desired lecithin analog, on careful and extensive drying, could be obtained in an anhydrous form on the basis of elemental analysis. On standing in air for a few hours the melting point was lowered a few degrees (but remained sharp) and a correct analysis for a monohydrated form was obtained. Another phosphonate-containing lecithin analog, 2-octadecyleicosylphosphonylcholine, previously has also been obtained in an anhydrous form (11).

EXPERIMENTAL PROCEDURES[2]

2-Hexadecoxy-3-octadecoxypropylphosphonylcholine

2-Hexadecoxy-3-octadecoxypropylphosphonic acid (ref 8; 317 mg, 0.50 m mol) and choline iodide (600 mg) were stirred together in a mixture of dimethylformamide (25 ml) and anhydrous pyridine (10 ml) at 43C for 20 min. To the almost clear solution was added *p*-toluenesulfonyl chloride (2.0 g) and the mixture was stirred at 40C for 48 hr.

The reaction mixture was concentrated in vacuo to about 4 ml (bath temperature, 35-40C); the product was precipitated by addition of acetonitrile (35 ml) and filtered. The yellowish solid was dissolved in a minimal volume of chloroform and reprecipitated with 15 vol. of acetone. The yield of product at this point was 320 mg. In chloroform-trifluoroacetic acid (9:1) on silica gel G plates the material was found to consist mainly of the desired product (Rf 0.33) with traces of the starting phosphonic acid and of unidentified phosphorus-containing contaminants.

The crude product (290 mg) was dissolved in chloroform (10 ml) and trifluoroacetic acid (0.5 ml) was added. The solution was evaporated to dryness, redissolved in chloroform, and reevaporated thoroughly. The product was dissolved in 5 ml of chloroform and applied to a 20 × 190 mm 3:1 silicic acid-celite (30 g) column, which had previously been activated by washing with methanol followed by an excess of chloroform.

The column was washed with 300 ml of chloroform, 300 ml of chloroform-methanol (9:1), and 600 ml of chloroform-methanol (3:1), all of which eluted only impurities. Further washing with 2100 ml chloroform-methanol (3:1) eluted the product in an almost pure form; yield, 208 mg (68% of crude product applied to column). This material was dissolved in chloroform (10 ml), pyridine (0.5 ml) was added, and the solution evaporated thoroughly to dryness. The residue was dissolved in chloroform (5 ml) containing one drop of pyridine, filtered through a medium-porosity sintered glass funnel, and warm acetone (10 ml) was added. The solution was boiled for 5 min, allowed to crystallize slowly at room

[2]TLC was performed on silica gel G plates; phosphorus-containing spots as well as all organic substances were visualized with the spray reagent of Dittmer and Lester (21), which has previously been shown to stain phosphonate-containing compounds (8). Microanalyses were performed by Schwarzkopf Microanalytical Laboratory, Woodside, New York 11377.

temperature and then at 5C, and finally filtered off and dried in vacuo. The product was recrystallized once more to give 197 mg (55%) of analytically pure white product, m.p. 193.5-194.5C. To obtain a correct analysis for the monohydrate form it was necessary to dry the product at 40C at 1 mm over P_2O_5 for 48 hr. Found: C, 68.41; H, 12.48; N, 1.83; P, 3.99. Calculated for $C_{42}H_{88}NO_5PH_2O$(736.163): C, 68.52; H, 12.32; N, 1.90; P, 4.21.

The product was chromatographically homogeneous in chloroform-trifluoroacetic acid (9:1; Rf 0.33) and in chloroform-methanol-water (65:25:4; Rf 0.53). Its infrared spectrum is shown in the figure.

1,2-Dipalmitoyl-sn-glycero-3-[2'-(trimethylammonium)ethylphosphonate]

2-(Trimethylammonium)ethylphosphonic acid monohydrate (ref 20; 0.74 g, 4 mmole) was added to a stirred suspension of phosphorus pentachloride (2.50 g, 12 mmole) in anhydrous alcohol-free chloroform (5 ml). The mixture was heated to reflux for 10 min and then stirred at room temperature for 30 min. Volatile material was removed in vacuo at 30–35C and the crystalline residue kept <0.1 mm at 50C for 1 hr to remove traces of phosphorus oxychloride.

The dried residue was suspended with stirring in a mixture of anhydrous pyridine (2.5 ml) and chloroform (15 ml) and stirred at 0–5C while a solution of *sn*-glycerol-1,2-dipalmitate[3] (ref 22; $[\alpha]_D^{25}$-3.0°; 1.12 g, 2 mmole) in chloroform (10 ml) and pyridine (2.5 ml) was added dropwise during 1 hr; residual dipalmitin was washed in with chloroform (3 × 4 ml). After 1 hr at 0–5C the mixture was stirred at room temperature for 20 hr.

The reaction mixture was again cooled to 0–5C and water (1 ml) was added with vigorous stirring, which was continued at room temperature for 2 hr. Solvents were removed in vacuo at 40C. The residue was extracted first with 0.15N HCl (120 ml), chloroform (25 ml) and isopropanol (50 ml). The aqueous layer was washed with 3 × 100 ml chloroform; the combined chloroform extracts were dried over $MgSO_4$, filtered and evaporated. The residual material was dried overnight at 35C < 1 mm.

A silicic acid-celite (3:1) column (4 × 26 cm tube) was prepared, containing 130 g of adsorbent previously activated by washing with methanol on a Buchner funnel (3 × 100 ml) and then freed of methanol by repeated washing with chloroform. The crude phosphonate lecithin in chloroform (25 ml) was applied to the column, which was washed successively with 350 ml chloroform, 1.6 liters chloroform-methanol (9:1), and 2.5 liters chloroform-methanol (4:1). The column effluents were monitored by TLC in chloroform-methanol-water (65:25:4). After about 1.3 liters chloroform-methanol (4:1) had been collected, the pure product began to appear and was completely eluted after 2.5 liters. Prior elutes contained unreacted dipalmitin, chlorodipalmitin, a variety of low-MW phosphorus-containing compounds, and the isomeric compound of unknown structure discussed above. [This substance had Rf 0.53 in chloroform-methanol-water (65:25:4); calculated for the anhydrous phosphonate lecithin: C, 66.72; H, 11.47; N, 1.94; P, 4.30. Found: C, 66.81; H, 11.30; N, 1.94; P, 4.10; m.p. 200–201 C, $[\alpha]_D^{25}$ + 6.1°. Its infrared spectrum is very similar to that of the product but differs in having a moderately weak but sharp absorption at 1620 cm^{-1}.]

The slightly off-white product, obtained by evaporation of the 1.3–2.5 liters of chloroform-methanol (4:1) eluate, was dissolved in a mixture of tetrahydrofuran (25 ml), chloroform (10 ml), methanol (6 ml) and water (4.5 ml) and decolorized by passage through a short column of Amberlite MB-3 previously equilibrated with the same solvent. The product, after evaporation of solvent, was obtained in an analytically pure form by a final crystallization from warm chloroform-acetone; its infrared spectrum was virtually identical with that given by Baer and Stanacev (7) for the dimyristoyl compound. After drying at 40C and < 0.1 mm over phosphorus pentoxide overnight, the m.p. was 201.5–202 C; $[\alpha]_D^{25}$ + 9.6° (c, 0.3 in alcohol-free chloroform). Calculated for anhydrous 1,2-dipalmitoyl-*sn*-glycero-3-[2'-(trimethylammonium)ethylphosphonate]: C, 66.72%; H, 11.47%; N, 1.94%; P, 4.30%. Found: C, 66.42%; H, 11.36%; N, 2.16%; P, 4.03%. After exposure to air at room temperature overnight, or on mixing with acetone containing a few drops of water and drying < 0.1 mm at room temperature overnight over sulfuric acid, the m.p. was 196.5–197C. Found: C, 65.44; H, 11.47. Calculated for monohydrate, C, 65.27; H, 11.23.

The material was chromatographically homogeneous on TLC in chloroform-methanol-water (65:25:4); Rf, 0.48. The compound was readily separable from the Rf 0.53 substance in an artificial mixture. The yield of pure product eluted from the column was 9.9%; how-

[3] D-$\alpha\beta$-Dipalmitin according to the older Fisher-Baer nomenclature.

ever, another 17.3% could be obtained from intermediate fractions (contaminated with the Rf 0.53 product) by rechromatography. The Rf 0.53 product was formed to the extent of 4.1%. About 38% of unreacted dipalmitin was recovered from the column.

ACKNOWLEDGMENT

This work was supported by grant no. AM-07699-04, National Institutes of Health.

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[Received May 23, 1968]

Metabolism of Lipids in Rat Testes: Interconversions and Incorporation of Linoleic Acid Into Lipid Classes

MASAMI NAKAMURA and **O. S. PRIVETT**, The Hormel Institute, University of Minnesota, Austin, Minnesota 55912

ABSTRACT

Studies are reported on the mode of incorporation of linoleic acid into lipid classes of testicular lipids. 1-^{14}C-linoleic acid was injected into the testes of adult rats of the Sprague-Dawley strain. Groups of animals were killed at 1, 3, 6, 12, 24 and 48 hr after injections of the radioactive linoleic acid. The testes of each animal and livers of some animals were excised. Fatty acid and lipid class composition of the extracted lipids of the testes of each animal were determined as well as the distribution of radioactvity in these compounds. Radioactive linoleic acid and fatty acids derived from it by interconversion and catabolism were incorporated into all the lipid classes. Incorporation of linoleic acid into the lipid classes was much faster than its interconversion or catabolism to other fatty acids. The importance of the fatty acid pool in the mode of incorporation of the fatty acids into the lipid classes is demonstrated.

INTRODUCTION

INTERRELATIONSHIPS IN LIPID synthesis in mammalian tissue have received a great deal of attention and enzymatic steps in pathways for the net synthesis of common phosphatides and triglycerides have been fairly well defined (3,11,14,29). Not so clear is the mode by which fatty acids are preferentially incorporated into these compounds. Diglycerides per se have been shown to be intermediates in the synthesis of triglycerides and phosphatides (14); fatty acids apparently may be preferentially incorporated into phosphatides via acyl transferases (15,16,17, 18,24). Patton et al. (21, 22, 23) postulated that in lactating mammary tissue phosphatidylcholine serves as an intermediate in triglyceride synthesis in order to explain the preferential incorporation of short chain fatty acids into milk triglycerides. However, fatty acid composition and positional arrangement (structure) of individual lipids generally do not conform to patterns that are common to each other as might be expected from consideration of synthetic pathways defined through studies on the mode of their interconversions (3,7,11,14,29). Recently (13) we demonstrated that relatively large changes may occur in fatty acid composition and lipid classes more or less independently of each other in testicular lipids indicating that turnover of acyl chains and skeletal moieties of triglycerides and phospholipids occur at different rates. Studies on the metabolism of testicular lipids have been devoted mainly to the mode of interconversion of fatty acids (1,4,5,12,19) and the effect of nutritional (1,8,13) and hormonal deficiencies (9,10,20) on lipid class and fatty acid composition. The present study was undertaken to provide basic data for further studies along these lines as well as for general information on the mode of lipid synthesis in the testes.

EXPERIMENTAL PROCEDURES

Materials and Methods

1-^{14}C-Linoleic acid was obtained from Tracerlab Inc., Waltham, Mass., methylated with diazomethane (27) and purified by argentation TLC. Radio gas chromatography (6) showed that all the radioactivity was associated with methyl linoleate in the final preparation. The free acid obtained via saponification and acidification had an activity of 3.5 m C/mM. For injections, the free acid was emulsified with a mixture of equal parts saline and rat serum; a 50 μl aliquot of this emulsion, the amount used for injections, had an activity of 1.4×10^5 c.p.m. Analysis of the radioactive linoleic acid in the emulsion showed that it remained unchanged and was stable at least for the period of the experiment.

Radioactivity was measured by scintillation counting with a Packard Tri-Carb Model 3002 dual channel scintillation spectrometer with a scintillation solution consisting of PPO-POPOP in toluene or in dioxane-water solution described by Snyder (28). The latter solution was used for analysis of material recovered from chromatoplates in radio TLC. Counting efficiency for carbon-14 was 85% in the toluene solution and 71% in the dioxane-water solution. Values were not cor-

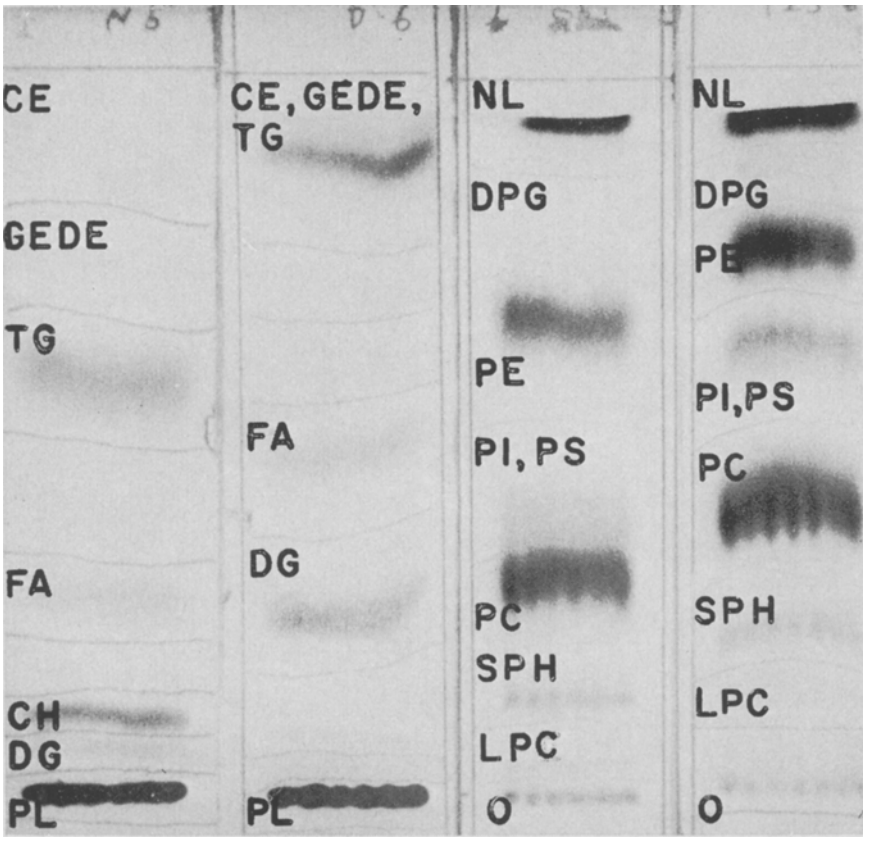

FIG. 1. TLC of rat testicular lipids. Plate 1 developed with petroleum ether-ethyl ether-acetic acid (80:20:1); plate 2 with petroleum ether-ethyl ether-methanol-acetic acid (90:20:5:2); plate 3 with chloroform-methanol-acetic acid (65:25:4:8); plate 4 with chloroform-water-acetic acid (25:15:-2:4). CE = cholesteryl esters, GEDE = glyceryl ether diesters, TG = triglycerides, FA = fatty acids, DG = diglycerides, PL = polar lipids, NL = neutral lipids, PE = phosphatidyl-ethanolamine, PS = phosphatidylserine, PI = phosphatidylinositol, PC = phosphatidylcholine, SPH = sphingomyelin, LPC = lysophosphatidylcholine, O = origin (unknown).

rected to 100% efficiency because results obtained in the different solutions were not compared.

Distribution of radioactivity among the lipid classes was determined by scintillation counting of bands of the components separated by TLC. The bands were scrapped directly from plates into vials of scintillation solution for counting. Fractionation of the lipids was carried out on four separate 5 × 20 cm chromatoplates containing a 0.25 cm layer of Silica Gel H (Brinkman Instruments Inc., Des Plaines, Illinois) as illustrated in Fig. 1. The positions of the bands were detected by exposing the plate to iodine vapors for just sufficient time to make them visible. The bands were marked and then the plates were placed in a chromatographic jar in a current of nitrogen for about 10 min to evaporate most of the iodine. Four different solvent systems were employed in order to separate the major components completely from each other and provide duplicate checks on all components. Plate 1 in Fig. 1 was developed in petroleum ether–ethyl ether–acetic acid (80:20:1) for analysis of cholesteryl esters (CE), glyceryl ether diester (GEDE), triglycerides (TG), free fatty acid (FFA), cholesterol (CH), diglyceride (DG) and polar lipids (PL) as a group. Plate 2 was developed with petroleum ether–ethyl ether–methanol–acetic acid (90:20:5:2). It was used mainly for separation of the diglyceride fraction, but it also provided a check on the total of TG + CE + GEDE, FA and the PL fraction. Cholesterol separated with the diglyceride in this plate, but generally it had little activity, as measured on Plate 1, and could be disre-

garded. Plate 3 was developed with chloroform–methanol–water–acetic acid (65:25:4:8). This plate was used mainly for phosphatidylethalolamine (PE) which separates completely in this solvent system; it also served to check Plate 4. The system used in Plate 4 consisted of chloroform–methanol–water–acetic acid (25:15:2:4). It has been described by Skipski et al. (25, 26) and its widely used for the separation of polar lipids.[1] Overall recoveries of radioactivity of approximately 96% were consistently obtained by the above technique. Specific activities were calculated from the amount of each lipid class determined on another aliquot of the sample by quantitative TLC by the charring-densitometry technique (2,13,20).

Fatty acid composition was determined with an F & M Scientific Corp. Model 1650 flame ionization gas chromatograph on methyl esters of the total lipid and lipid classes prepared by interesterification with methanol–HCl. Nitrogen was used as the carrier gas, and separations were carried out on 6 ft. × ¼ in. column packed with Gas Chrom P containing 16% EGSS-X (Applied Science Laboratory, State College, Pa.). Percent composition was determined on the basis of the direct proportionalities of the peak areas measured by triangulation. The average of triplicate analyses of standard mixtures of reference fatty acids of composition similar to those distributed by the NIH (purchased from the Lipids Preparation Laboratory of The Hormel Institute) agreed with the known composition within a maximum of ± 2.6% and ±5.4% relative error for the major and minor components, respectively.

Radio gas chromatography of methyl esters was carried out essentially by the collection technique described by Dutton (6) with an F & M Model gas chromatograph equipped with a thermal conductivity detector and a 6 ft. × ¼ in. column packed with Gas Chrom P containing 16% EGSS-X (applied Science Lab, State College, Pa.). The collected samples were counted with a Packard Tricarb Model 3002 scintillation counter. Recoveries of radioactivity by this technique were approximately 80%. Specific activities were calculated from the amount of each component determined by GLC via pentadecanoic acid added to the esterification mixture as an internal standard. Application of this technique to a number of standard radioactive fatty acids gave values that agreed within ±1.5% between duplicate analyses.

Animals

Adult male rats of the Sprague-Dawley strain of 200–225 g were obtained from the Hormone Assay Laboratory, Chicago, Ill. The animals were housed in individual metal cages and fed ad lib. a semi-synthetic diet consisting of 30% vitamin test casein, 50% sucrose, 4% cellulose[2], 4% mineral mix[3], 2% vitamin mix[4] and 10% safflower seed oil. At the end of three weeks the animals were divided into six groups and one testicle of each animal in each group was injected with 50 μl of the 1–^{14}C–linoleic acid emulsion (1.4 × 10^5 c.p.m.) in a sequential experiment. The animals were killed by exsanguination by withdrawal of the blood from the aorta, and the testes and livers were excised and frozen on dry ice. Groups were killed at 1, 3, 6, 12, 24 and 48 hr after injection of radioactivity. The testes of each animal in each group were decapsulated and weighed. The lipid was then extracted twice with chloroform–methanol (2:1) and once with a 1:2 ratio of these solvents, recovered in the usual manner as previously described (2,13,20). The distribution of the radioactivity and analyses of the lipid classes and fatty acids were determined as described above.

RESULTS

The percent distribution of radioactivity among the lipid classes of the testes and general data on the different groups of animals are presented in Table I. These results show that 53.1% of the injected radioactivity were recovered in the testicular lipids of the animals in the first group and 30.2% were recovered from the testes of the last group of animals (48 hr after the injections of radioactive linoleic acid). In an accessory experiment, it was found that 80% of the injected radioactivity could be recovered in the testicular lipids of animals killed 15 min after injection of radioactive linoleic acid. No radioactivity was

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[1] PC = phosphatidylcholine, PI = phosphatidylinositol, PS = phosphatidylserine. SPH = sphingomyelin, LPC = lysophosphatidylcholine, DPG = diphosphatidylglycerol, NL = neutral lipid, PL = polar lipid.

[2] Non-nutritive cellulose, alphacel from Nutritional Biochemicals Corp., Cleveland, Ohio.

[3] Wesson modified, Osborne-Mendel salt mix, from General Biochemicals, Chagrin Falls, Ohio.

[4] Vitamin mix, consisting of 0.25% vitamin A acetate crystals, 0.017% vitamin D_2 concentrate (400,000 U.S.P. μ/g), 1.70% alpha-tocopherol, 0.06% *i*-inositol, 5.0% choline chloride 0.0085% menadione, 0.0325% *p*-aminobenzoic acid, 0.43% niacin, 0.13% riboflavin, 0.035% pyridoxine HCl, 0.13% thiamine HCl, 0.43% calcium pantothenate, 0.001% biotin, 0.045% folic acid, 0.0002% vitamin B_{12}, and 90.77% casein diluent.

TABLE I

Percent Distribution of Radioactivity Among the Testicular Lipids of Animals Injected Intratesticularly With 1-^{14}C-Linoleic Acid

Time period (hr)		1	3	6	12	24	48
No. of animals		5	5	5	6	6	6
Testis wt. (g)		1.49±0.03[a]	1.57±0.06	1.51±0.03	1.64±0.15	1.59±0.03	1.53±0.07
% Lipid		2.7 ±0.1	3.0 ±0.1	3.1 ±0.1	3.2 ±0.1	3.2 ±0.04	3.3 ±0.1
Recovery of radioactivity %		53.1 ±1.0	48.5 ±1.4	46.7 ±0.5	47.5 ±3.2	43.8 ±2.4	30.2 ±1.4
Neutral lipids (% distribution of radioactivity)							
	Composition[b] (wt. %)						
CE	....	tr[c]	tr	0.5 ±0.04	0.6 ±0.04	0.9 ±0.1	1.0 ±0.2
GEDE	....	tr	tr	tr	tr	0.7 ±0.1	0.9 ±0.1
TG	9.5	5.7 ±0.2	5.0 ±0.04	7.9 ±0.8	6.1 ±0.6	12.2 ±0.5	11.0 ±1.5
FA	3.0	20.2 ±1.1	20.6 ±0.9	19.0 ±0.9	20.0 ±1.0	19.5 ±0.8	16.0 ±0.8
DG	1.8	8.0 ±0.4	6.9 ±0.3	6.4 ±0.2	7.1 ±0.3	6.3 ±0.4	6.0 ±0.4
Chol.	.....	tr	tr	tr	tr	tr	tr
Total Neutral lipids (NL)		35.9 ±1.2	34.4 ±1.7	33.5 ±1.5	35.2 ±1.5	40.4 ±0.7	36.2 ±0.6
Polar lipids (% distribution of radioactivity)							
DPG	....	1.4 ±0.2	2.4 ±0.2	1.5 ±0.3	1.5 ±0.2	1.4 ±0.3	1.6 ±0.1
PE	27.4	11.7 ±0.3	14.2 ±0.3	16.3 ±0.5	19.1 ±0.6	17.6 ±0.5	18.6 ±0.4
PI+PS	4.3	3.2 ±0.2	4.2 ±0.1	5.2 ±0.6	7.6 ±0.4	8.0 ±0.2	9.3 ±0.3
PC	27.4	44.3 ±1.0	42.1 ±1.6	40.7 ±0.3	33.2 ±0.5	30.3 ±0.6	31.4 ±0.6
Sph	....	0.6 ±0.02	0.8 ±0.02	0.5 ±0.1	0.6 ±0.1	1.1 ±0.1	1.4 ±0.1
Lyso		1.8 ±0.2	0.6 ±0.1	0.5 ±0.1	0.5 ±0.1	0.3 ±0.3	0.6 ±0.1
Total polar lipids (PL)		64.0 ±1.2	65.5 ±1.7	66.5 ±1.5	62.8 ±1.5	59.3 ±0.7	63.8 ±0.6

[a] M±SE.

[b] Major radioactive components.

[c] Trace = under 0.4%.

found in the lipids of the livers or testicle not injected with 1–^{14}C–linoleic acid during the course of the experiment. Thus, no data are reported on these organs and it may be concluded that the radioactive linoleic acid was metabolized in the testicle into which it was injected. Further evidence to this effect was the relative constancy of the percentage distribution of the radioactivity among the lipid classes in view of the fact that approximately 70% of it was dissipated over the course of the experiment. This observation also indicates that linoleic acid undergoes transformations among the lipid classes in a very orderly pattern, because the percentage composition (by weight) of the components does not change during the period of the experiment. The changes that did occur in the percentage distribution of the radioactivity among the lipid classes were probably due primarily to conversion of linoleic acid to other fatty acids that were transformed among the lipid classes and catabolized at different rates. More information of the mode of transformations of the fatty acids among the lipid classes was obtained from a consideration of the data on the specific activities of the lipid classes presented in Figures 2 and 3.

The fatty acid fraction had the highest specific activity, as expected. The specific activity of this fraction gradually decreased as the fatty acids were catabolized and new fatty acids entered the pool to dilute the radioactivity. The concentration of the radioactivity (specific activity) in PC and DG followed the same pattern as the fatty acid fraction but since these compounds contained no radioactivity originally, the specific activity apparently reached a maximum at some time prior to 1 hr, the time at which the first group of animals was killed. The concentration of radioactivity in PE appeared to reach a maximum next among the lipid classes at between 6 and 12 hr. The peaks in the concentrations of the radioactivity of the other lipid classes came at approximately 24 hr. Analyses were not made at short enough time intervals to indicate the maxima of the specific activities with great precision. Nevertheless, it was apparent the specific activities reached maximal values first in DG and PC, then in PE and later in the other lipid classes.

The percentage distribution of the radioactivity among the fatty acids in the different

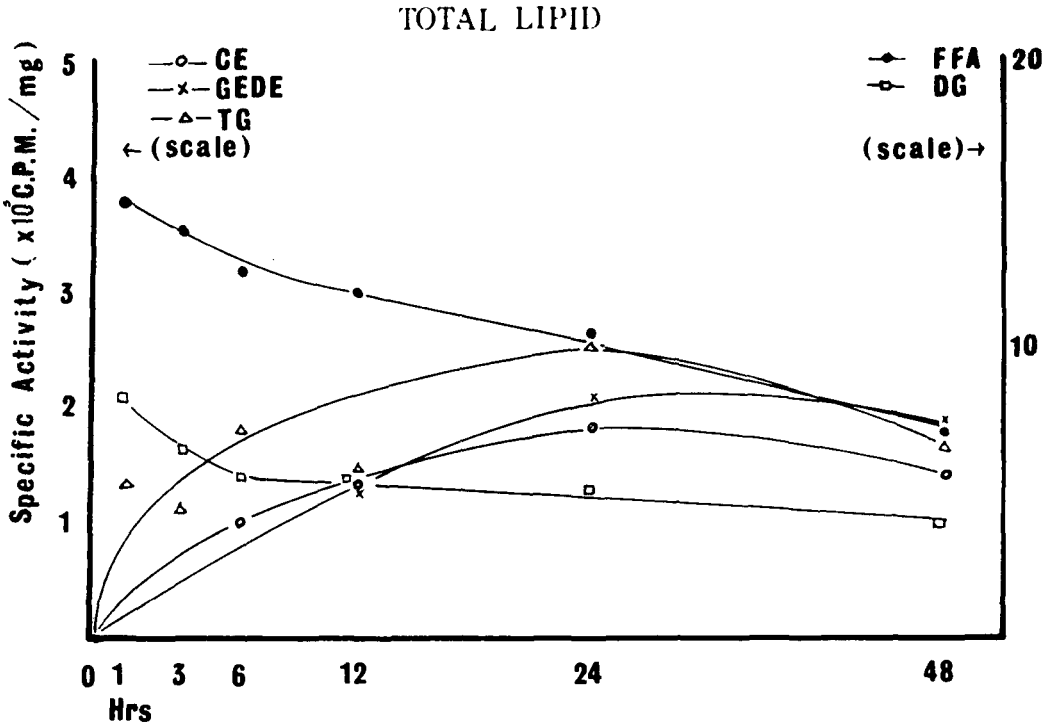

FIG. 2. Specific activities of neutral lipids of the testicular lipids of animals injected intratesticularly with 1-^{14}C-linoleic acid. CE = cholesteryl esters, GEDE = glyceryl ether diester, TG = triglycerides, FFA = free fatty acids, DG = diglycerides.

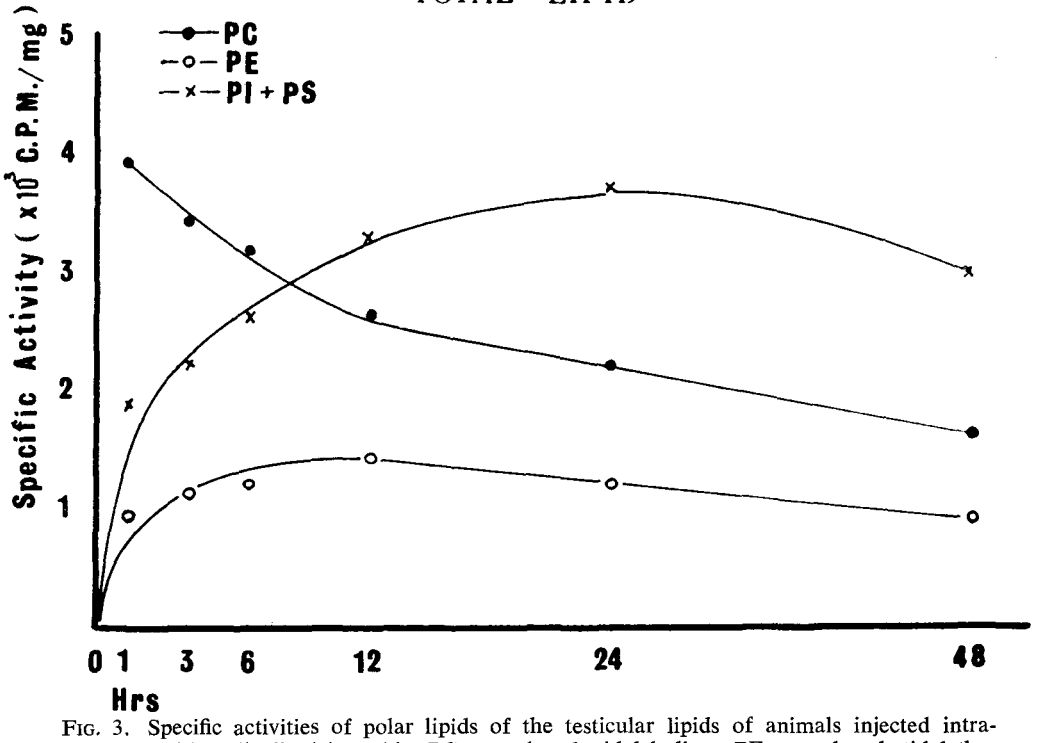

FIG. 3. Specific activities of polar lipids of the testicular lipids of animals injected intratesticularly with 1-^{14}C-linoleic acid. PC = phosphatidylcholine, PE = phosphatidylethanolamine, PI = phosphatidylinositol, PS = phosphatidylserine.

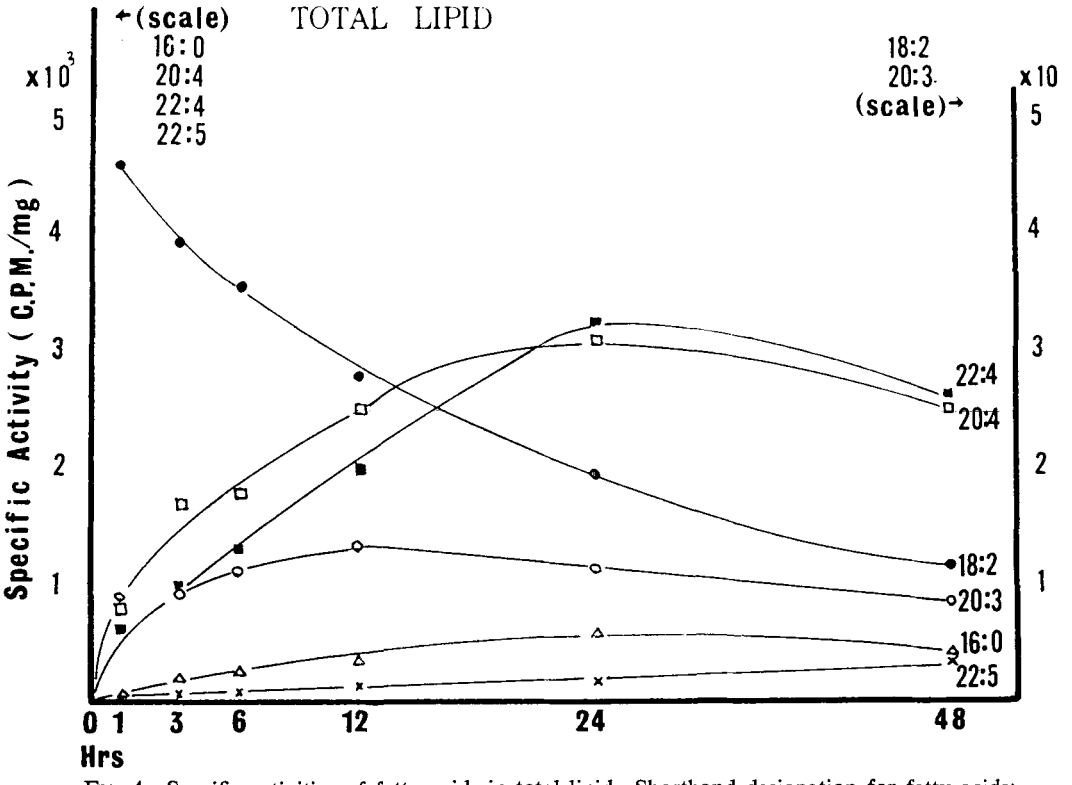

FIG. 4. Specific activities of fatty acids in total lipid. Shorthand designation for fatty acids; number before colon = number of carbon atoms in chain; number after colon = number of double bonds.

groups is summarized in Table II. As the percentage distribution of radioactivity of linoleic acid decreased that in the other fatty acids increased showing that linoleic acid underwent interconversion to other members of this family of acids. However, linoleic acid still contained the highest percentage of radioactivity even in the last group of animals in which all but approximately 30% of the radioactivity had been dissipated. The radioactivity in palmitic acid may be presumed to arise by de novo synthesis from radioactive acetate produced in the catabolism of radioactive acids.

More information on the interconversion and catabolism of the fatty acids was indicated from the plot of their specific activities in the total lipid (Fig. 4). Figure 4 shows the specific activities of the major fatty acids determined in the total lipid. No results were

TABLE II

Per Cent Distribution of Radioactivity Among the Fatty Acid of the Testicular Lipids of Animals Injected Intratesticularly With 1-^{14}C-Linoleic Acid

		% Radioactivity					
	Time period (hr)	1	3	6	12	24	48
	Composition (wt %)						
16:0	29.1±2.6	2.8[a]	3.4±0.2[b]	4.5[a]	6.0±0.2	11.2±1.1	13.0±0.8
18:2	6.1±0.4	81.8	75.2±0.5	71.5	60.1±0.9	42.7±1.8	35.3±3.5
20:3	1.5±0.1	3.9	4.1±0.4	4.9	5.8±0.2	5.9±0.3	6.0±0.5
20:4	15.3±1.6	3.5	8.5±0.3	8.7	12.8±0.5	17.0±1.1	20.1±1.3
22:4	2.7±0.02	0.6	0.9±0.3	0.9	1.6±0.1	3.2±0.3	4.3±0.3
22:5	20.8±1.8	0.2	0.3±0.1	0.4	0.9±0.1	2.3±0.3	4.1±0.3

[a] Pooled samples.

[b] M + SE.

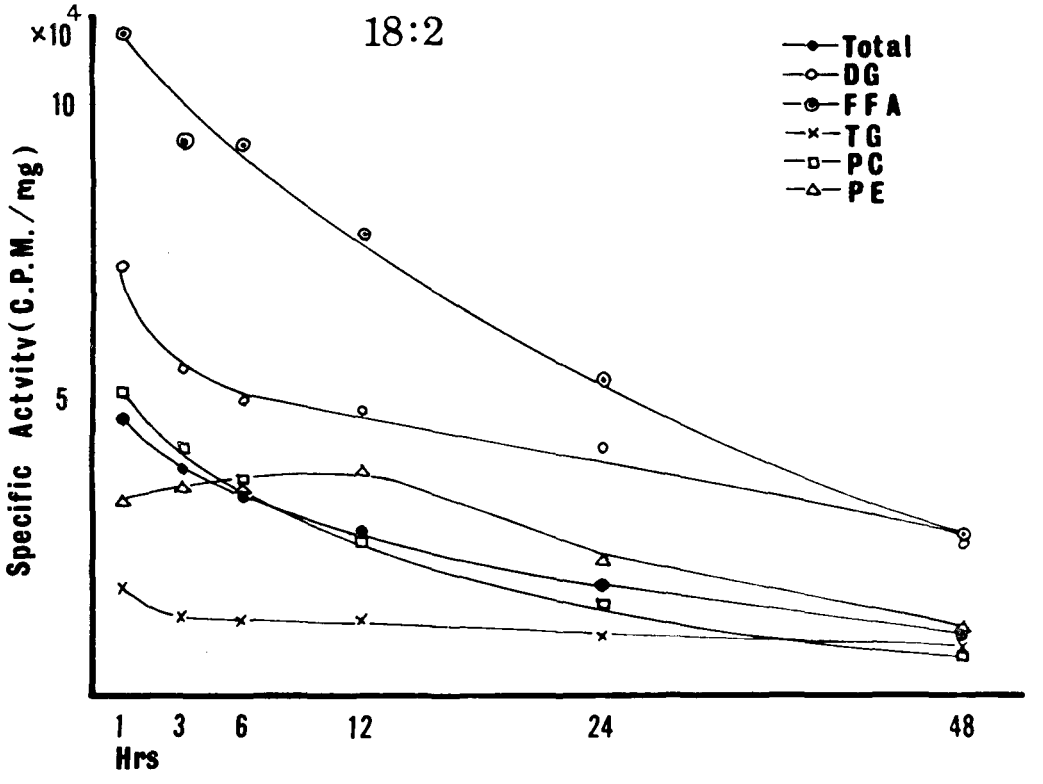

FIG. 5. Specific activities of linoleic acid (18:2) in the lipid classes. DG = diglycerides, FFA = free fatty acids, TG = triglyceride, PC = phosphatidylcholine, PE = phosphatidylethanolamine.

obtained on 18:3 (gamma linolenic) or 20:2 (11,14–eicosadienoic acid) members of the linoleic acid family, because in addition to being in small concentrations they could not be separated quantitatively for counting. Specific activities of the major fatty acids in PC, PE, TG and FFA fractions were also determined. These showed the same general pattern as for the total lipid except that there were some differences in the order of magnitude of the values for the different fatty acids in each lipid class. In general, the specific activities of the 22:4, 20:3 and 20:4 were highest in the polar lipids, reached maximal concentrations and were decreasing by the end of the experiment. The 16:0 and 22:5 were the lowest and, except for the 16:0 in TG, did not exhibit a peak in concentration of radioactivity. Conversions to 22:5 were relatively slow. Linoleic acid exhibited a decay curve in all of the lipid classes except PE where it appeared to peak at about 12 hr. (Fig. 5.) Since there was no radioactive linoleic acid in the lipid classes originally (except in the FA fraction) its specific activity reached a maximum in these compounds before the first group of animals was killed. Thus, the incorporation of linoleic acid into the lipid classes is much faster than its conversion to other fatty acids. The fatty acids derived from linoleic acid probably also were incorporated into the lipid classes very quickly but the rates of incorporation could not be determined because the measurement of their specific activities depended on their conversion from radioactive linoleic acid. However, the specific activities of all fatty acids containing radioactivity in the various lipid classes were determined. Typical of these data are those shown for arachidonic acid in Fig. 6. The specific activities of all of the fatty acids were highest in the free fatty acid fraction and next highest in DG (Fig. 6). There were some differences in the order of magnitude of the specific activities of the individual fatty acids from one lipid class to another but they were probably related to the rate of their interconversion; that of arachidonic acid was highest in PC of the polar lipids (Fig. 6).

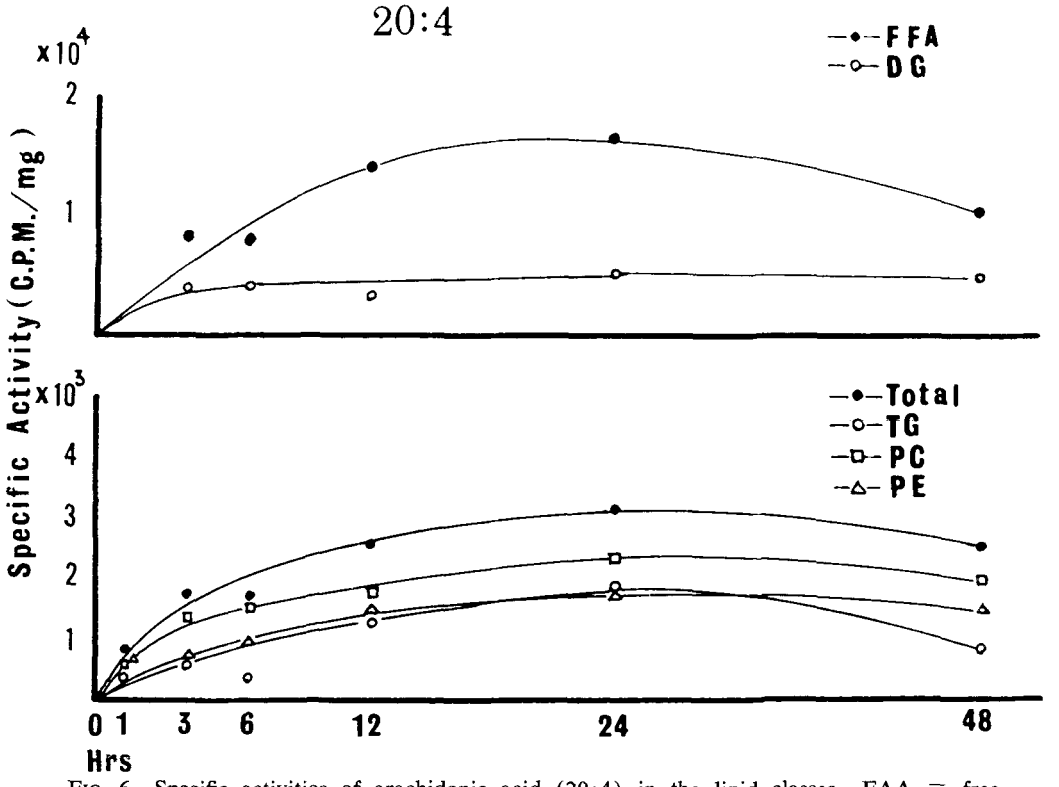

FIG. 6. Specific activities of arachidonic acid (20:4) in the lipid classes. FAA = free fatty acids, DG = diglycerides, TG = triglycerides, PC = phosphatidylcholine and PE = phosphatidylethanolamine.

DISCUSSION

The distribution of radioactivity among the entire spectrum of fatty acids shows that linoleic acid undergoes interconversion and is also catabolized. Evidence for catabolism is the presence of radioactive palmitic acid. The radioactivity in this fatty acid must arise by de novo synthesis from radioactive acetate derived from injected linoleic acid. These processes (catabolism and interconversion) go on more slowly than the incorporation of linoleic acid into the lipid classes. Evidence to this effect is that, in addition to its rapid incorporation into the lipid classes, linoleic acid contained most of the radioactivity (35%), remaining 48 hr after injection of the animals. The relatively slow interconversion of linoleic acid is not because this process is performed only in the liver. Several reports (4,12,19) demonstrate the interconversion of fatty acids by testicular tissue. In the present study the amount of radioactivity in the lipid of livers and the testicle not injected with radioactive 1-^{14}C-linoleic acid was insignificant, even after 48 hr. Thus, it may be concluded that interconversions and catabolism as well as incorporation of radioactive linoleic acid into the lipid classes occurs entirely in the testes in the present study.

It is well established that lipid in living tissue is in a dynamic state; turnover measured by changes in composition may be a fairly slow process varying from several months in some tissues to several days or weeks in more active metabolizing tissues. In the present work no measurable changes in composition of the lipid classes would be expected in the 48 hr period of the experiment. However, the individual reactions involved in the overall process of lipid metabolism appear to be rapid, judging by the fact that approximately 70% of the radioactivity was dissipated in a period of 48 hr, and the radioactive linoleic acid was incorporated into all the lipid classes in less than 1 hr.

The mode of incorporation of fatty acids into the lipid classes is important in determining composition and positional arrangement of the fatty acids. The present study indicates the importance of the fatty acid pool in these

processes. It may be calculated that 1 hr after injection of the radioactive linoleic acid, 6.1 $\mu\mu$ curies of this acid is present as free fatty acid (in the fatty acid pool). However, after 48 hr 13.3 $\mu\mu$ c of fatty acid derived from linoleic acid is present in the lipid classes. If, as generally assumed, interconversions and catabolism involve fatty acid-CoA derivatives as obligatory intermediates in these processes, then linoleic acid must have been released from the lipid classes to account for the synthesis of these acids. This, and the general pattern of distribution of radioactivity in Table I, indicate that the acyl chains of the lipid classes are in dynamic equilibrium with the fatty acid pool. Accordingly, fatty acids may be taken from the pool and directed preferentially into not only the lipid classes but also into specific positions in the molecules of these compounds. Molecular species as well as fatty acid composition of the lipid classes may be regulated via the action of specific enzymes in these reactions. Changes in the percentage of distribution of the radioactivity among the lipid classes were relatively small compared to the large change in total amount of radioactivity during the course of the experiment. These changes are believed to occur mainly as a result of conversions of linoleic acid to other fatty acids that are distributed in a different manner and rate from that of linoleic acid.

The precise pathways whereby fatty acids are incorporated into the lipid classes have not been completely elucidated. Fatty acids may undergo transformations among the lipid classes in accordance with the Kennedy pathway (14), but this process appears to apply more to the mode of net synthesis of lipid than to a dynamic state of equilibrium. Whether the greater incorporation of radioactive linoleic acid into phosphatidylcholine rather than triglycerides represents a deviation from the Kennedy pathway or a ramification of it is not known. It appeared that diglycerides were intermediates in these reactions because of their high specific activity. However, the radioactivity in the diphosphatidylglycerol fraction which should also contain phosphatidic acid appeared to be too low for phosphatidic acid to be an intermediate in these reactions in accordance with the Kennedy pathway. The amount and concentration of radioactivity in the lysophosphatidylcholine fraction also appeared to preclude it as an intermediate for existence of an important acyl transferase system (7,15,17,18) or as a product of the release of fatty acids into the fatty acid pool. However, if the incorporation and equilibrium processes involved only the 2 position of lysophosphatidylcholine, no large amount of radioactivity would accumulate in this molecule. Thus, although the present study provides evidence for the importance of the fatty acid pool in dynamic equilibrium with the lipid classes, much further experimentation is required to delineate the reactions involved in the general process of lipid synthesis in the testes.

ACKNOWLEDGMENT

This investigation was supported in part by PHS research grants AM 04942 and HE 08214 from the National Institutes of Health, Public Health Service. B. D. Westra gave technical assistance.

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[Received May 29, 1968]

The Stereochemistry of Enzymic Hydration and of Chemical Cleavage of D-(+)-*cis*-12,13-Epoxyoleic Acid (Vernolic Acid)

L. J. MORRIS and **M. L. CROUCHMAN**, Division of Biochemistry,
Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford, England

ABSTRACT

The absolute optical configurations of (+)-*threo*-12,13-dihydroxyoleic acid, derived by enzymic hydration of endogenous vernolic acid in crushed *Vernonia anthelmintica* seed, and of (−)-*threo*-12,13-dihydroxyoleic acid, derived by acetolysis-hydrolysis of vernolic acid, have been determined. The absolute configuration of the (+)-enantiomer is L-12,D-13-dihydroxyoleic acid and, as the parent vernolic acid is known to be D-12,D-13-epoxyoleic acid, the stereochemistry of the enzymic hydration is thus shown to involve attack by hydroxyl at the 12 position with inversion at that position. Chemical cleavage of vernolic acid, on the other hand, involves preferential nucleophilic attack, with inversion, at the 13 position.

INTRODUCTION

VERNOLIC ACID ((+)-*cis*-12,13-epoxyoleic acid) has been shown to occur in the seed oils of numerous species of the plant families Compositae, Euphorbiaceae, Onagraceae, Valerianaceae and Dipsacaceae (1). The enantiomer of vernolic acid, namely (−)-*cis*-12,13-epoxyoleic acid, also occurs naturally in the seed oils of a number of the Malvaceae (2). In *Vernonia anthelmintica* seed there is an epoxy acid hydrating enzyme which, on incubation of the crushed seed under moist conditions, cleaves the endogenous (+)-vernolic acid to give optically pure (+)-*threo*-12,13-dihydroxyoleic acid (3,4).

The absolute optical configuration of (+)-vernolic acid was recently established (5) as being D, i.e., 12-*S*,13-*R* in the Cahn-Ingold-Prelog system (6). It was then reasoned that (−)-*threo*-12,13-dihydroxyoleic acid, derived as the predominant enantiomer by acetolysis-hydrolysis of (+)-vernolic acid, was D-12,L-13-dihydroxyoleic acid, i.e., 12-*S*,13-*S*. Consequently, the (+)-*threo*-dihydroxy acid produced by enzymic cleavage was considered to be the L-12,D-13-enantiomer, i.e., 12-*R*,13-*R* and it was suggested that the enzymic attack must be at the 12 position, resulting in inversion at that position.

This paper describes the direct determination of the absolute optical configurations of the (+)- and (−)-enantiomers of *threo*-12,13-dihydroxyoleic acid. The reactions involved in this determination are summarized in Figure 1. They consist of the production of pairs of positionally isomeric hydroxy,tosyloxyoleates from vernolic acid, by epoxide cleavage with toluene-*p*-sulphonic acid, and from (+)- or (−)-*threo*-12,13-dihydroxyoleic acid, by partial tosylation with toluene-*p*-sulphonyl chloride. The stereochemistry of the starting materials and of these reactions is such that the pair of isomers produced from vernolic acid must be D-12-hydroxy,L-13-tosyloxy- and L-12-tosyloxy,D-13-hydroxyoleic acids, whereas the pair produced from the *threo*-dihydroxy acid must be either D-12-hydroxy,L-13-tosyloxy- and D-12-tosyloxy, L-13-hydroxy-oleic acids or the enantiomeric pair. Thus, each of the positional isomers obtained from vernolic acid must be optically identical with the corresponding positional isomer from one of the *threo*-dihydroxy acids and enantiomeric with the like isomer from the other *threo*-dihydroxy acid. The absolute configurations of the two positional isomers derived from vernolic acid are known so that, if it can be determined which of these isomers is which, then the configurational problem is solved. Reaction of tosyloxy groups with $LiAlH_4$ or $LiBH_4$ in boiling tetrahydrofuran results in the hydrogenolysis of the tosyloxy group. When this reaction is applied to the individual hydroxy, tosyloxyoleate isomers the corresponding hydroxyoleyl alcohols are produced. These, then, can be characterized by mass spectrometry (7) after conversion to the corresponding ketostearates.

The results obtained by this procedure are in accord with our previous deductions (5) as to the mechanism and stereochemistry of chemical cleavage of vernolic acid and of the hydration of vernolic acid by the enzyme present in *Vernonia* seed.

EXPERIMENTAL PROCEDURES

Methyl D-(+)-*cis*-12,13-epoxyoleate (methyl vernolate) was isolated from the mixed esters derived from *V. anthelmintica* seed oil by adsorption column chromatography, as described previously (8). (+)-*threo*-12,13-Dihydroxyoleic acid was obtained by incu-

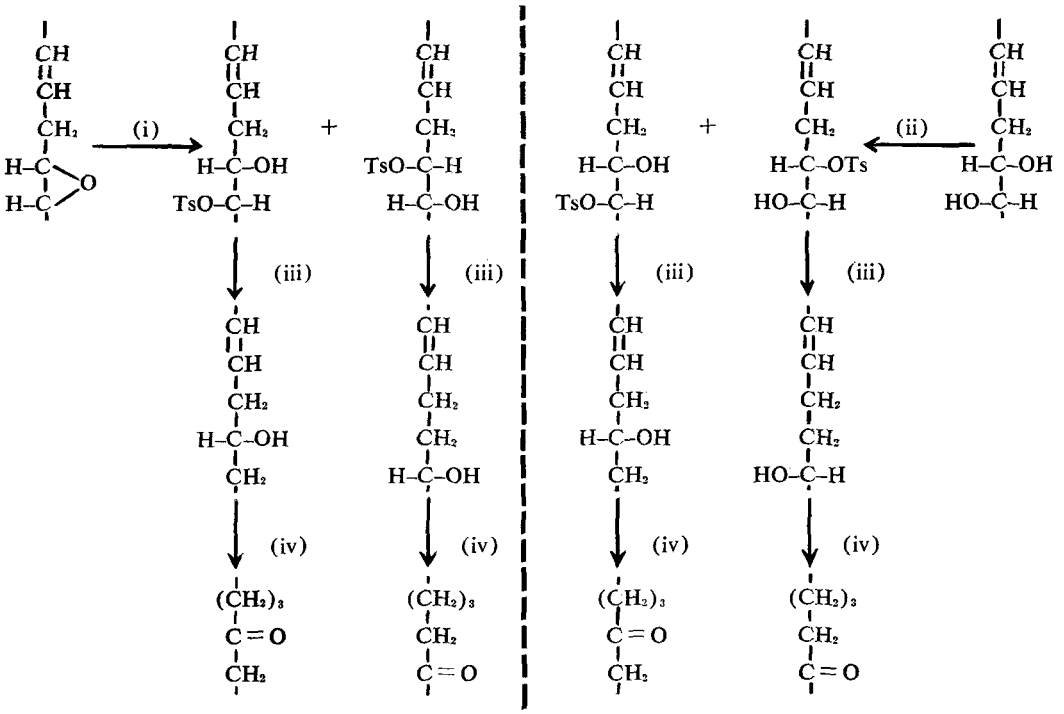

FIG. 1. Summary of reactions used in the determination of the absolute optical configuration of *threo*-12,13-dihydroxyoleate. Reactions are: (i) epoxide cleavage with toluene-*p*-sulphonic acid in diethyl ether; (ii) partial tosylation with toluene-*p*-sulphonyl chloride in pyridine; (iii) reduction with $LiAlH_4$ in tetrahydrofuran; (iv) catalytic reduction followed by oxidation with chromium trioxide in acetic acid.

bation of wetted, ground *V. anthelmintica* seed for three days at 25 C, in an atmosphere of nitrogen saturated with water (3). Total lipids were then extracted with chloroform–methanol (2:1), recovered, dissolved in ether, and free fatty acids were extracted into 10% aqueous KOH. The crude free acid fraction was recovered, esterified with diazomethane and fractionated by column chromatography on silica gel. The pure methyl *threo*–12,13–dihydroxyoleate, eluted with 30% ether in light petroleum, had $[\alpha]_{546.1\ m\mu}^{27°} = +20.3°$ (c = 1.0% in EtOH) and was evidently optically pure (2,3,9).

(–)–*threo*–12,13–Dihydroxyoleic acid was obtained from methyl vernolate by acetolysis with hot acetic acid followed by hydrolysis with 10% methanolic KOH, essentially as described by Gunstone (10). After two crystallizations from acetone, the chemically pure dihydroxyoleic acid was esterified with diazomethane and the methyl ester had $[\alpha]_{546.1\ m\mu}^{27°} = -7.6°$ (c = 2.5% in EtOH). Further recrystallization of the acid to obtain the pure optical enantiomer ($[\alpha]_D = -19°$), as described by Hopkins and Chisholm (2,9) was not attempted and this partially racemic ester was used for the subsequent work.

Preparation of Methyl Threo-12(13),13(12)-Hydroxy,tosyloxyoleates

Methyl vernolate (1.0/g) was dissolved in anhydrous diethyl ether (5 ml) and added to a solution of toluene–*p*–sulphonic acid (0.78/g) in anhydrous ether (15 ml). The reaction mixture was shaken for 30 min, allowed to stand at room temperature overnight, diluted with ether, washed twice with 10% aqueous KOH and then with water to neutrality. The solution was dried and evaporated to yield 940 mg (63%) of a pale yellow oil, which consisted almost entirely of hydroxy,tosyloxyoleates, as judged by TLC.

Methyl (+)– or (–)– *threo*–12,13–dihydroxyoleate (240 mg) was dissolved in pyridine (1ml) and added to a solution of toluene–*p*–sulphonyl chloride (143 mg) in pyridine (1 ml); the mixture was shaken for 30 min and allowed to stand at room temperature overnight. The mixture was diluted with water, extracted with diethyl ether and the extract

TABLE I

Characteristics of Products From Methyl Vernolate and (+)- and (−)-*Threo*-12,13-Dihydroxyoleates

Starting material	(+)-vernolate		(+)-*threo*-diOH-oleate		(−)-*threo*-diOH oleate	
[α] 546.1mμ	+5.4°		+20.3°		−7.6°	
Hydroxy, tosyloxyoleate	upper	lower	upper	lower	upper	lower
[α] 546.1mμ	−6.5°	−18.8°	−8.0°	+15.6°	+2.1°	−5.8°
Hydroxyoctadecenol						
[α] 546.1mμ	+1.6°	+2.4°			−0.3°	+0.8°
Ketostearate isomer	13	12			13	12

All optical rotations were measured in ethanol except that of methyl vernolate, which was in chloroform. The specific rotations of the partially racemic (−)-dihydroxyoleate and its products were all approximately one third of the specific rotations of the (+)-dihydroxyoleate and the products from it or from methyl vernolate, or both.

The terms "upper" and "lower" refer to the positionally isomeric hydroxy,tosyloxyoleates having, respectively, greater and lesser mobility on argentation TLC, as described in the text, and to the hydroxyoctadecenol and ketostearate products derived from them.

was washed twice each with water, dilute HCl, water, dilute aqueous KOH and finally with water to neutrality. The ether solution was dried and evaporated to yield ca. 300 mg of a pale yellow oil which, according to TLC, contained ca. 60% of hydroxy,tosyloxyoleate and also ditosyloxyoleate and unchanged dihydroxyoleate. The hydroxy,tosyloxyoleate fraction was isolated by preparative TLC.

Separation of Hydroxy, Tosyloxyoleate Positional Isomers

On normal unimpregnated silica gel layers, no appreciable separation of the two positionally isomeric *threo*-12(13),13(12)-hydroxy, tosyloxyoleates could be achieved, even on multiple development. However, on silica gel impregnated with silver nitrate there was clear separation of the mixture into two bands, after double development with diethyl ether–light petroleum (1:1). As in the case of cleavage with hydrogen halides (5), the two positional isomers derived from methyl vernolate were not formed in equal amounts and the less mobile isomer on argentation–TLC was the major component (60–70%). The two positional isomers derived from dihydroxyoleate were in approximately equal amounts.

The individual isomers from each product mixture were isolated by preparative argentation TLC, approximately 30 mg of mixture being separated on each 200 × 200 × 1 mm layer. The plates were developed twice with ether-light petroleum (1:1), the separated components were located under ultraviolet light after spraying with dichlorofluorescein and were eluted from the scraped-off adsorbent with pure diethyl ether. The separated positional isomers were all completely pure as judged by analytical argentation TLC.

Hydrogenolysis of Hydroxy, Tosyloxyoleate Isomers

Each of the pure hydroxy,tosyloxyoleate isomers (10–50 mg) was dissolved in anhydrous tetrahydrofuran (3 ml) and $LiAlH_4$ (50 mg) was added. The mixture was refluxed overnight, cooled and the complex then decomposed by the addition of excess dilute sulphuric acid. The product was extracted into ether, washed with water and the solvent was removed to yield a colourless oil. TLC indicated that each product was almost entirely a hydroxyoleyl alcohol, having suffered hydrogenolysis of the tosyloxy group and reduction of the ester group.

Preparation of Ketostearates

The hydroxyoleyl alcohols were each hydrogenated at atmospheric pressure in ethyl acetate solution over Adam's platinum oxide catalyst. The white solid hydroxystearyl alcohol products (5–10 mg) were each dissolved in a solution of 5% chromium trioxide in glacial acetic acid (1 ml) and shaken vigorously for 5 min. The solutions were then diluted with water, extracted with ether and the extracts washed thoroughly with water. The recovered products were each esterified with diazomethane and the ketostearates purified by preparative TLC.

Physical Methods

Optical rotations were measured in a 2.0 cm cell with an ETL/NPL Automatic Polarimeter (Type 143A), as solutions (concentrations 0.5–5.0%) in ethanol.

Mass spectra were obtained on an AEI MS12 instrument using the direct insertion sample probe technique and were interpreted on the basis of the published work of Ryhage and Stenhagen (7) and in comparison with the mass spectra of pure authentic samples of methyl 12– and 13–ketostearates.

RESULTS

A summary of the various products and of their specific rotations is provided in the Table I.

The two hydroxy,tosyloxyoleates derived from methyl vernolate both had the same sign of rotation, unlike the corresponding

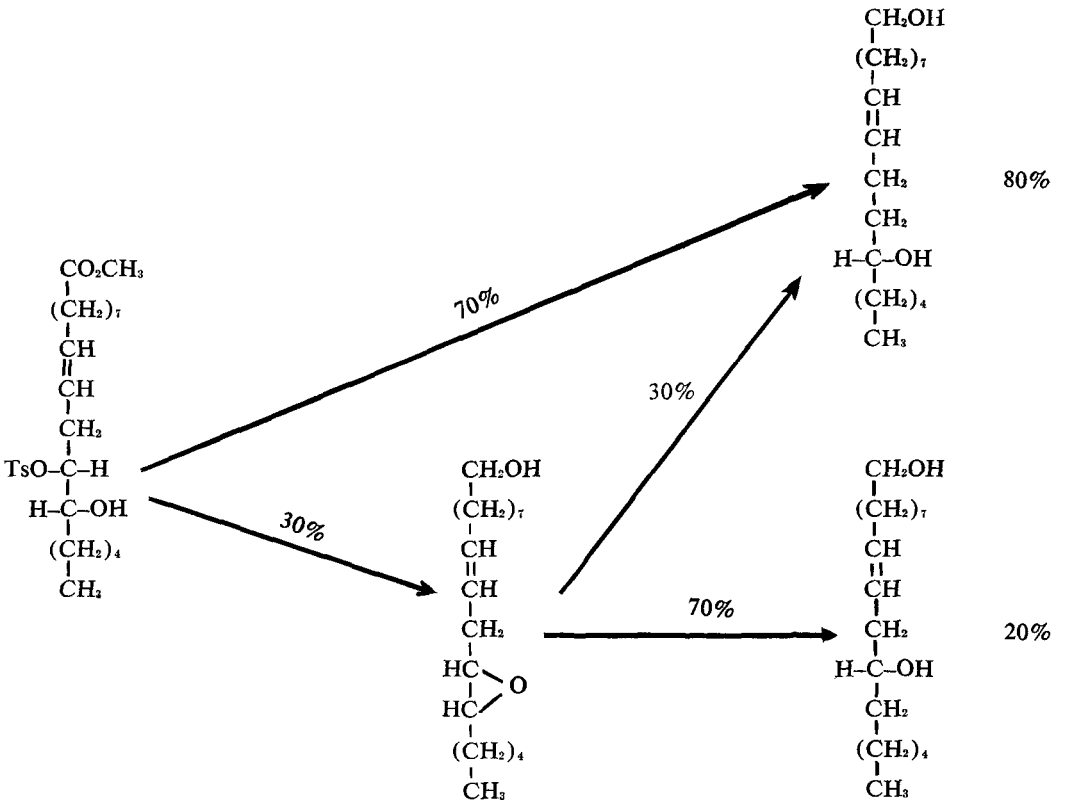

FIG. 2. Summary of postulated concurrent reactions and proportions of products in reduction of 13-hydroxy,12-tosyloxyoleate with $LiAlH_4$, in boiling tetrahydrofuran.

chlorohydroxyoleate derivatives which had opposite rotations (5). In each case, however, one isomer must be the D–12–hydroxy,L–13–tosyloxy– or –chloro–oleate and the other isomer must be the D–13–hydroxy,L–12–tosyloxy– or chloro–oleate, from the known stereochemistry of epoxide ring opening by acids (11). This is confirmed by the fact that the 12– or 13–hydroxyoleyl alcohols, produced on hydrogenolysis of the tosyloxy group from each isomer, were both dextrorotatory as is ricinoleyl alcohol ($[\alpha]_D$ = +3.0° in $CHCl_3$ (5)), which is known to be D–12–hydroxyoleyl alcohol.

The methyl ketostearate derived from the vernolate hydroxy,tosyloxy derivative of lower mobility on argentation TLC was proved by its mass spectrum to be almost entirely 12-ketostearate, with only a very small proportion of 13-ketostearate present. The other vernolate derived ketostearate, from the upper or more mobile hydroxy,tosyloxyoleate, was largely 13–ketostearate but it also contained a substantial proportion (ca. 20%) of 12–ketostearate. The position of the hydroxy group in the two derivatives from (–)–12,13–dihydroxyoleate was determined in the same way and with identical results from the mass spectra. The presence of these positionally isomeric ketostearate impurities in what should be pure, positionally homogenous products, is discussed below.

We have therefore now established that the less mobile (lower) hydroxy,tosyloxyoleate is the 12-hydroxy-isomer and if it is laevorotatory, it is the D–12–hydroxy,L–13–tosyloxy enantiomer. Similarly, the more mobile (upper) isomer is 13–hydroxy,12–tosyloxyoleate which if it is laevorotatory is the D–13–hydroxy,L–12–tosyloxy enantiomer.

Thus, of the products derived from (–) *threo*–12,13–dihydroxyoleate by partial tosylation, the less mobile isomer was laevorotatory and hence was D–12–hydroxy,L–13–tosyloxyoleate, identical with the corresponding vernolate product. The more mobile isomer, on the other hand, was dextrorotatory and therefore enantiomeric with the corresponding vernolate product, i.e., it was L–13–hydroxy, D–12-tosyloxyoleate. The configuration of the

substituents in both of these derivatives was D–12, L–13 and the parent (–)–dihydroxyoleate was thereby proved to be D–12,L–13–dihydroxyoleate, i.e., 12–*S*,13–*S*.

The (+)–*threo*–12,13–dihydroxyoleate produced by enzymic hydration of (+)–vernolic acid must be L–12,D–13–dihydroxyoleate, i.e., 12–*R*, 13–*R* and this was confirmed by the formation from it of D–13–hydroxy,L–12–tosyloxyoleate and L–12–hydroxy,D–13–tosyloxyoleate, these being respectively laevo– and dextrorotatory and hence identical and enantiomeric with the corresponding derivatives from vernolate.

DISCUSSION

Although the pair of positionally isomeric hydroxy,tosyloxyoleates showed no sign of separation on normal silica gel, they were quite readily separated from each other on silver nitrate impregnated silica gel. This separation implies a reduction in the silver ion-complexing ability of the double bond of the more mobile isomer, shown to be the 13–hydroxy,12–tosyloxyoleate. This is probably due to steric hindrance toward complexing exerted by the bulky tosylate group close to the double bond or to delocalization of the π– electrons of the double bond by this group or both.

The nucleophilic attack on the epoxy group of vernolic acid during reduction with $LiAlH_4$ has been shown (5) to occur predominantly at the 13 position to give the 12-hydroxy derivative. It was then argued that any nucleophilic reagent would likewise preferentially attack the 13 position. The present work has verified this in that the reaction of methyl vernolate with toluene–*p*–sulphonic acid gave unequal proportions of the hydroxy,tosyloxy isomers and the major product (60–70%) was again the 12–hydroxy isomer. The configurations previously assigned to the two chlorohydrin isomers from methyl vernolate (5) are thus confirmed.

One initially puzzling feature of the present work was the presence of the other isomer in each of the individual ketostearates, particularly of 12–ketostearate as a substantial impurity (ca. 20%) in the 13–ketostearate product. These findings were unexpected because each of the separated hydroxy,tosyloxyoleate isomers, from which these products were derived by seemingly straightforward reactions, was judged to be greater than 95% pure by argentation TLC. The reason for this is believed to be that the basic conditions of the hydrogenolysis reaction caused elimination of toluene–*p*–sulphonic acid from some proportion of the hydroxy, tosyloxyoleate thereby regenerating epoxyoleate which was then reductively cleaved in the normal way to give both 12– and 13–hydroxy isomers in approximately a 2:1 ratio. If some 30% of the 13–hydroxy,12–tosyloxyoleate went via this "epoxide pathway" on hydrogenolysis, as summarized in Figure 2, the product would consist of about 80% of the expected 13-hydroxyoleyl alcohol and 20% of the 12–hydroxy isomer. These proportions are close to the actual composition obtained, as determined by mass spectrometry of the derived ketostearate. On the same basis, the 12–hydroxy,13–tosyloxyoleate would give 90% of the desired 12–hydroxyoleyl alcohol and only about 10% of the 13–hydroxy isomer as impurity.

This work has demonstrated that the enzymic hydration of endogenous D–*cis*–12,13–epoxyoleic acid in crushed *Vernonia* seeds produces L–12,D–13–dihydroxyoleic acid. On the assumption that the stereochemistry of enzymic and chemical opening of the epoxide ring must be the same, namely inversion at the position of nucleophilic attack (and it is difficult to visualize any other stereochemistry), then the site of hydroxyl attack by the enzyme must be at the 12 position, as previously predicted (5). This is now being verified directly by incubation of crushed *V. anthelmintica* seeds in the presence of $H_2{}^{18}O$. Preliminary results indicate that the ^{18}O isotope is enriched exclusively in the 12–hydroxyl group of the (+)–*threo*–12,13–dihydroxyoleic acid product (13).

ACKNOWLEDGMENTS

W. Kelly, of this Laboratory, performed the mass spectral analyses and provided interpretations of the spectra.

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[Received May 1, 1968]

Comparison of Lipoprotein Analysis by Agarose Gel and Paper Electrophoresis With Analytical Ultracentrifugation[1]

R. P. NOBLE, The Sharon Research Institute, Sharon Hospital, Sharon, Connecticut, **F. T. HATCH, J. A. MAZRIMAS**, Bio-Medical Division, Lawrence Radiation Laboratory, University of California, Livermore, California, **F. T. LINDGREN, L. C. JENSEN** and **G. L. ADAMSON**, Donner Laboratory, University of California, Berkeley, California

ABSTRACT

A comparison has been made of human serum lipoprotein analysis by agarose gel and paper electrophoresis with a standard method of analytical ultracentrifugation. Samples were obtained from 28 patients with various disorders of lipoprotein metabolism. Correspondence was shown between the following electrophoretic and ultracentrifugal fractions: β and S_f 0-20; pre-β and S_f 20-400; α_1 and total HDL. The deviations observed with the electrophoretic methods, though sizable, were smaller than the usual clinically significant abnormalities. Semiquantitative application is therefore justified. Agarose gel electrophoresis is slightly more difficult than paper electrophoresis, but gives improved resolution of pre-β- and β-lipoproteins and better densitometric scans. Evidence was also presented that the agarose method, when used in conjunction with ultracentrifugation, may be a valuable research technique for the study of lipoprotein properties.

INTRODUCTION

THE RELATIONSHIPS BETWEEN serum lipoprotein fractions measured with the analytical ultracentrifuge and with paper electrophoresis were reported in 1967 by Hatch et al. (1). A newer electrophoretic technique for the separation of the serum lipoproteins has been developed which employs agarose gel as the supporting medium (2). With this technique there is improved separation of pre-β- from β-lipoproteins, and the stained zones appear on a transparent, durable plastic strip. This preparation permits unusually precise scans of the strips with a densitometer. Pre-β bands of slow, intermediate and fast mobility have been noted. This paper compares the results of lipoprotein analyses in 28 human sera by analytical ultracentrifugation, paper electrophoresis and agarose gel electrophoresis.

MATERIALS AND METHODS

Blood samples, containing 0.1 mg/ml of thimerosal (Eli Lilly & Co., Indianapolis, Ind.) as a preservative, were obtained in the morning from 28 nonfasting patients, aged 30-55 years, with various disorders of lipoprotein metabolism. These patients were under observation or treatment with diet or drugs. All had been previously identified as having hypercholesterolemia or hypertriglyceridemia or both. At the time these blood samples were drawn their lipoprotein levels ranged from normal to markedly elevated. No hypolipoproteinemic patients were studied. The blood was allowed to clot for 2 hr at room temperature; the clot was freed from the wall of the tube, and the blood cells were sedimented by centrifugation at 800 *g* for 10 min.

Analytical ultracentrifugation, after preliminary separations of low and high density lipoproteins in the preparative ultracentrifuge, was performed by the method of Ewing et al. (3). Moving boundary flotation rates of the major S_f 0-12 lipoprotein component were measured and converted to standard conditions (4).

Paper electrophoresis of lipoproteins was performed in barbital buffer at pH 8.6 and ionic strength 0.1, containing 1% (w/v) human albumin, by the method of Lees and Hatch (5). Staining was carried out for 6 hr at 37C in a saturated solution of oil red O in 60% ethanol.

Agarose electrophoresis was performed by the technique of Noble (2). In brief, 0.25 g of agarose (Bio-Rad Laboratories, Inc., Richmond, Calif.) were brought to boiling in 50 ml of 0.05 M barbital buffer at pH 8.6. The solution was cooled to 45C and 1 ml of a 25% solution of human serum albumin (Pentex, Inc., Kankakee, Ill.) was added. Bovine albumin fraction V may also be used. Four milliliters of the agarose mixture were pipetted onto the surface of 35 × 185 mm strips of Cronar *C-41* polyester film (Photo Products Dept., E. I. duPont de Nemours Co., Wilmington, Del.). The agarose was spread from edge to edge and within 1 cm of the ends of the strips. A chrome steel rod 2 × 20 mm was placed transversely near one end of the agarose to form the sample trough.

[1]Presented at the AOCS Meeting, Washington, D.C., April 1968.

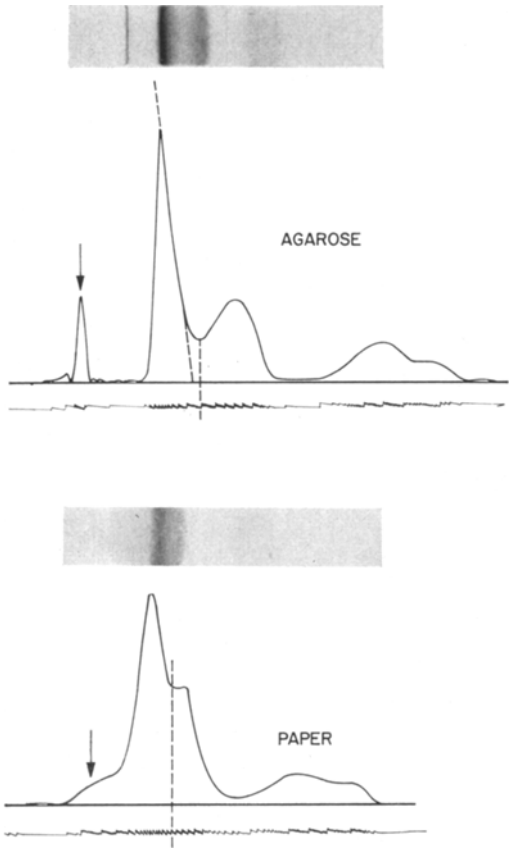

FIG. 1. Agarose gel and paper electrophoresis were performed on the same serum sample obtained from a hypercholesterolemic (Type II) (6) subject 4 hrs after a fat load of 2 g/kg. The test meal caused the appearance of a chylomicron zone at the origin (vertical arrows) and produced a densely stained pre-β zone which is helpful in this photographic illustration.

Note that the drive speed for the electrophoretic strip was reduced to one half the chart speed, i.e., from 8 cm/min to 4 cm/min.

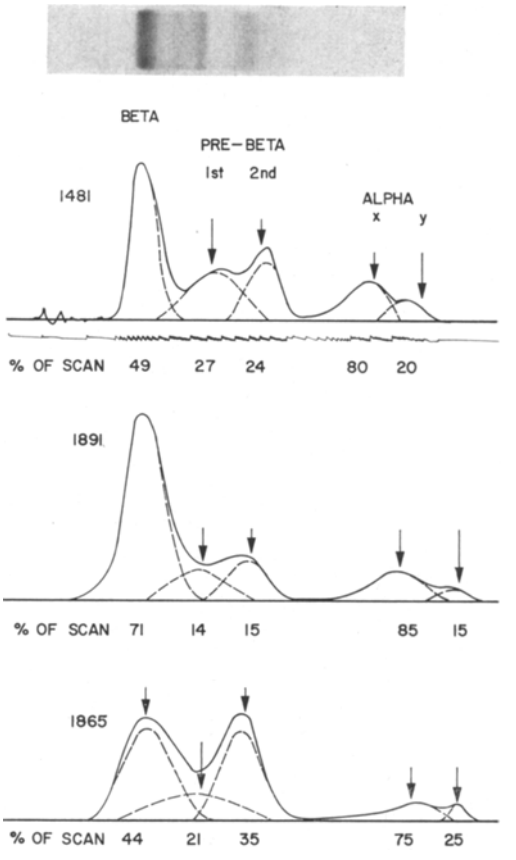

FIG. 2. Agarose gel electrophoresis. The electrophoretic strip and upper scan (1481) are from an unusual sample illustrating two visibly distinct pre-β zones. The dashed lines in the densitometric scans show the three Gaussian components into which the β + pre-β complex and the two components into which the α_1 zone were resolved with the duPont Curve Resolver. The curve resolver gives the percentage of area which is contributed by each of the three components to the β + pre-β complex and by each of the two components to the α_1 complex. These values are recorded beneath the Analytrol integrator traces as % of scan.

The lower scan (1865) illustrates a sample in which the large pre-β components distort the leading edge of the β zone so that projection of its slope to the baseline would give a grossly high value for β zone area. In such cases the β zone limit was placed at the same point as the pre-β determined by the perpendicular dropped from the "valley." Drive speed for the electrophoretic strip was reduced as in Figure 1.

Samples were prepared by transferring 50 μl of serum in a micropipet into a test tube in a water bath at 45C; 25 μl of warm agarose solution were added. Fifty microliters of the mixture, containing 33 μl of serum, were applied in the troughs after the gel had set and the rods had been removed with a magnet.

Five strips were placed in a horizontal electrophoretic cell (E-C Apparatus Corp., Philadelphia, Pennsylvania). A constant current of 10 mA per strip was applied for 2 hr at approximately 250 v. The migration of the α_1-lipoprotein zone was 70-75 mm.

The strips were fixed in 85% ethanol containing 5% acetic acid (v/v) for 45 min. The strips were dried in an oven at 85C for 15 min and were stained in the above solution of oil red O for 18 hr at 37C.

The electrophoretic zones, β, pre-β, and α_1

were measured on duplicate paper strips and single agarose strips for each sample in the Spinco Analytrol Model RB (Beckman Instruments, Inc., Fullerton, Calif.) with automatic integration. The methods for demarcating each lipoprotein fraction on paper and agarose are shown in Figure 1.

For agarose, the leading boundary of the β zone was determined by projecting to the baseline the best straight line describing the descending slope; pre-β, a line was drawn perpendicular to the baseline from the lowest point between the β and pre-β peaks. For paper, the trailing boundary of the β-lipoprotein was determined by projecting to the baseline the best straight line describing the ascending slope; the leading boundary was placed halfway between two lines perpendicular to the baseline located at the first and second perceptible deviations from the straight line describing the descending slope; pre-β extended from the aforementioned leading boundary of the β zone to the point where the tracing approached the baseline closely.

The scans for agarose electrophoresis were also analyzed with a Model 310 Curve Resolver (Instrument Products Div., E. I. duPont de Nemours Co., Wilmington, Del.) as shown in Figure 2. Results obtained by the two methods were similar, except for additional information from the resolver method to be discussed below. Correlation and regression relationships between the densitometric scans and the ultracentrifugal analyses were calculated with a Control Data Corp. 6600 computer.

RESULTS AND DISCUSSION

A comparison of the results obtained by electrophoresis in agarose and by analytical ultracentrifugation is presented in Figure 3 in the form of scatter diagrams. The correlation coefficients between both agarose and paper electrophoresis and ultracentrifugation are summarized in Table I. In addition the number of cases out of the total of 28 in which the data lay within ± 30% of the regression line calculated by the method of least squares is tabulated for each fraction.

The electrophoretic mobilities on paper of

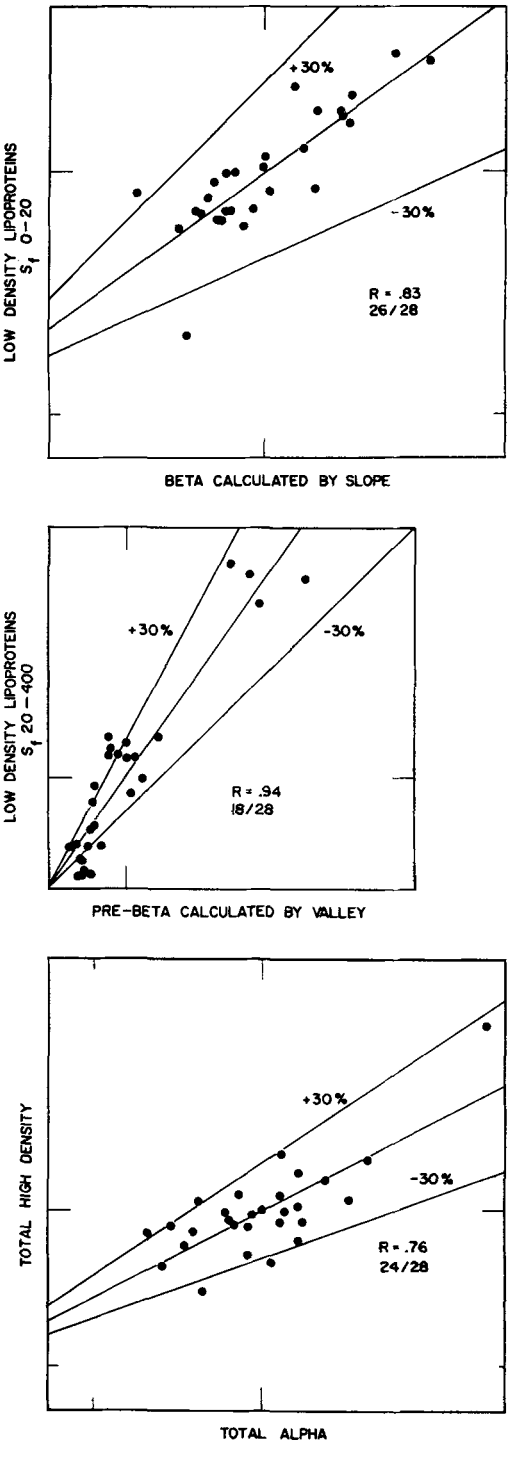

FIG. 3. Scatter diagrams plotted by a California Computer Corp. X-Y plotter. The abscissae record the areas of the electrophoretic zones and the ordinates record the ultracentrifugal values for the appropriate lipoprotein classes. The central line is the regression line calculated by the method of least squares and the other lines deviate by ± 30% of the Y value. Correlation coefficients are given by R. The fraction numerators are the number of cases whose data lie within ± 30% of the regression line, and the denominator (28) is the total number of cases studied.

TABLE I

Comparison of Lipoprotein Separation in Agarose Gel and on Filter Paper with Ultracentrifugal Sf Classes by Correlation and Regression Analysis in 28 Cases[a]

		β vs. Sf 0–20	Pre-β vs. Sf 20–400	β + Pβ vs. Sf 0–400	α vs. HDL
Correlation Coefficients	Agarose	0.83	0.94	0.79	0.76
	Paper	0.85	0.88	0.76	0.76
Number of Cases ± 30% of Regression Line	Agarose	26	18	27	24
	Paper	25	19	27	25

[a]Paper electrophoresis was performed in duplicate. The mean value is shown. A single strip was analyzed in the agarose technique.

the major lipoprotein fractions that can be isolated by preparative ultracentrifugation have been previously established (5). The relationships correspond to those in Table I, which are confirmed in this study for both types of electrophoresis by means of analytical ultracentrifugation.

The correlation coefficients and the degree of adherence to regression for agarose and for paper electrophoresis were in remarkably good agreement. It was noteworthy that when sizable deviations from the regression lines occurred, they were usually similar in direction and magnitude for both the agarose and paper methods. This suggests that the major deviations reflect differences in lipoprotein properties affecting depth of staining rather than errors of technique.

When the pre-β zones were small, as in normal subjects or patients with low levels of serum triglycerides, there was increased scatter of these low points around the regression line in both electrophoretic methods. Previously Hatch et al. reported that in one third of 32 normal subjects the pre-β zones on paper were too small and insufficiently separated from the β zone for satisfactory analysis (1). In our experience with the agarose technique pre-β zones are visible in all subjects, although at low levels the comparison with the ultracentrifugal data may be nearly as variable as in paper electrophoresis.

Study of the Analytrol scans of agarose gel electrophoresis strips with the duPont Curve Resolver showed that three Gaussian components were required to fit the envelope of the β- pre-β complex and two components for the α_1 zone (Fig. 2). The use of Gaussian components is supported by the densitometric scan of the normal β fraction in lipoprotein electrophoresis. Furthermore, when Margolis (7) isolated very low density (VLDL), low density (LDL) and high density (HDL) liproproteins by means of agarose gel filtration he found that the elution patterns could be described by Gaussian curves.

Occasionally electrophoretograms showing two distinctly visible bands in the pre-β region have been observed in normal and diabetic subjects and more often in patients who had been treated with clofibrate, 2 g/day, for at least 2 months (Fig. 2, pattern 1481). When the VLDL fraction was isolated by preparative ultracentrifugation and was subjected to agarose gel electrophoresis, a small band moving more slowly than the major pre-β band was frequently observed. The position of this slower band corresponded to the first pre-β component demonstrated with the curve resolver. Apparently the slower pre-β component overlaps the β zone in most serum patterns making difficult its visualization as a separate entity.

The two pre-β components revealed by the curve resolver were compared by correlation analysis serially with the ultracentrifugal classes ranging from S_f 0 to 400 (Fig. 4). The correlation profiles show that the first (slower) pre-β component correlated most strongly with the ultracentrifugal range S_f 12-50 whereas the second (faster) pre-β component correlated best with the range S_f 60-250. Thus in the VLDL range there is evidence for a positive correlation between electrophoretic mobility and ultracentrifugal flotation rate. A semiquantitative evaluation of the distribution of the lipoproteins within the very low density fraction can be made with the agarose method. This distribution has not been revealed by paper electrophoresis because of the poorer separation of pre-β and β lipoproteins.

Recently in a small series of selected human sera we have observed a strong inverse correlation between the relative mobility of the β zone in agarose electrophoresis and the corrected $S_f°$ rate of the LDL peak in the S_f 0-12 fraction. Under constant conditions of technique the relative mobility of the β zone should reflect variations in the net charge on the lipoprotein molecules or in the degree of coverage of the molecular surface by protein. The peak

$S_f°$ rate in the analytical ultracentrifuge is inversely related to the hydrated density, directly related to the molecular weight (8), and is probably related to the relative proportions of lipids and protein in the lipoproteins (9). Since significant differences in the peak flotation rate of LDL exist between normal men and women and in certain disease states (10), further study of the relationships between the electrophoretic and ultracentrifugal properties of lipoproteins is important.

DISCUSSION

Highly significant correlation coefficients were observed between both electrophoretic methods (agarose gel and paper) and analytical ultracentrifugation for the following fractions: β and S_f 0-20; pre-β and S_f 20-400; and α_1 and total HDL. From 85% to 96% of the individual determinations lay within ± 30% of the regression lines calculated from all of the data, except for greater scatter of pre-β values at very low (normal) levels. The degree of agreement of the electrophoretic methods with ultracentrifugation qualifies the former as semiquantitative. In general use, however, the clinically significant abnormalities of lipoprotein metabolism are of much greater magnitude than the observed deviations between the methods. It is important to note that the largest deviations were similar by both electrophoretic methods, suggesting that differences in lipoprotein properties rather than technical errors were responsible.

The relatively simple and inexpensive electrophoretic methods can be used on a semiquantitative basis in clinical and epidemiological studies. The agarose gel method is slightly more difficult to perform, but provides better resolution of pre-β- and β-lipoproteins and more satisfactory densitometric scans.

In addition to its usefulness for routine analyses, agarose gel electrophoresis appears to be a research tool for the study of lipoprotein properties. We have indicated its ability to estimate the distribution of lipoproteins within the VLDL fraction and interesting relationships between LDL electrophoretic mobility and ultracentrifugal $S_f°$ rate of the major S_f 0-12 component. Further study of potential research applications is in progress.

ACKNOWLEDGMENT

This work was performed under the auspices of the U.S. Atomic Energy Commission. It was supported in part by Grant 350 from the Northwest Chapter of the Connecticut Heart Association, and by U. S. Public Health Service Grants 5-RO1-HE-07220-6 and 5-RO1-HE-1882-14. Blood samples were provided by E. H. Strisower.

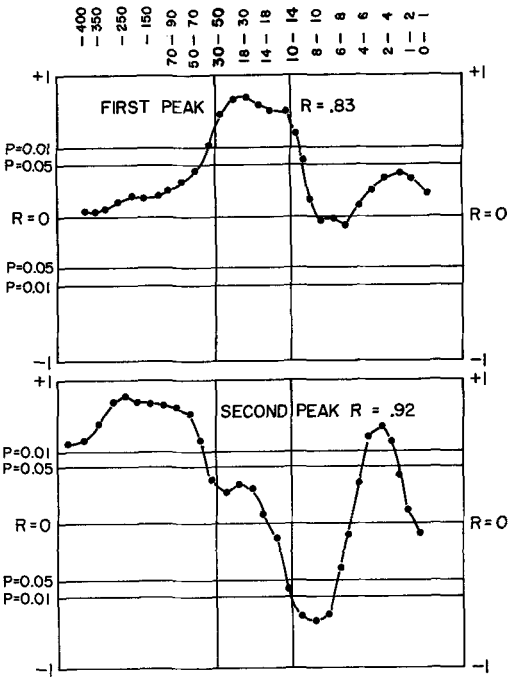

FIG. 4. Correlation profiles: correlation coefficients were determined for first and second pre-β components obtained with the curve resolver (Fig. 2) sequentially with each subclass of the ultracentrifugal S_f 0-400. Positive or negative correlation coefficients are plotted along the ordinate and the levels are shown outside of which the probability (P) of chance occurrence is less than 0.05 or 0.01.

The first (slower) pre-β component correlates significantly with the S_f 12-50 class and the second (faster) component correlates significantly with the S_f 60-250 class. The electrophoretic mobility of these VLDL subfractions thus appears to be directly proportional to the ultracentrifugal flotation rate.

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[Received July 24, 1968]

Screening for Serum Lipoprotein Abnormalities: Comparison of Ultracentrifugal, Paper and Thin-Layer Starch-Gel Electrophoresis Techniques[1]

LENA A. LEWIS, Research Division, Cleveland Clinic Foundation, Cleveland, Ohio 44106

ABSTRACT

Three methods for evaluation of serum lipoprotein abnormalities were compared: paper electrophoresis using buffer containing albumin, ultracentrifugation at d 1.21, and thin-layer starch-gel electrophoresis. Analyses by paper electrophoresis and by ultracentrifugation of 109 sera of patients with cholesterol levels between 78 and 1150 mg/100 ml showed that the electrophoretic procedure while not quantitative was an effective procedure for detecting and helping in classification of the type of serum lipoprotein abnormality. Paper electrophoresis is a valuable procedure in screening for lipoprotein abnormalities and can be used as a guide for additional studies. Thin-layer starch-gel electrophoresis did not always give results comparable to those of the other two procedures. In study of certain sera (for example, in pigeon serum and some myeloma sera) lipoprotein subfractions were more clearly resolved by starch-gel electrophoresis than by paper electrophoresis or ultracentrifugation. Ultracentrifugal and starch-gel electrophoretic techniques can be used effectively when more complete information is needed or when unusual materials are being studied.

INTRODUCTION

A SCREENING PROCEDURE to demonstrate abnormalities or to detect differences in serum lipoprotein patterns of humans or other species or strains must give clear resolution of the different types of serum lipoproteins and be sufficiently sensitive to demonstrate differences in their concentration. The results must be reproducible and the determination requires only small amounts of serum. A paper electrophoresis technique has been effectively utilized by Frederickson et al. (1) to determine the type of human serum lipoprotein pattern.

[1]Presented at the AOCS Meeting, Washington, D. C., April 1968.

EXPERIMENTAL

Methods

In this study three methods of evaluating serum lipoproteins have been used: (a) In the paper electrophoretic procedure of Lees and Hatch (2) a barbital buffer containing 1% albumin, and oil-red O stain for demonstration of the lipid components after electrophoresis are utilized. Bovine albumin was used instead of human material as originally described. (b) Ultracentrifugation at a density of 1.21 using NaCl-KBr by Lewis et al. (3) modification of the Gofman et al. procedure (4). At density 1.21 –S (0–10), –S (25–70) and –S (70–400) correspond approximately to HDL_{2+3}, S_f 0–20 and S_f 20–200, respectively. And (c) thin-layer starch-gel electrophoresis as described by Lewis (5), using the tris-borate double buffer system of Poulek (6), and oil-red O stain for lipids.

Materials

The sera of 109 patients in the post-absorptive state, with atherosclerosis, multiple sclerosis, multiple myelomatosis, xanthomatosis or excessive obesity were analyzed by paper electrophoresis and by ultracentrifugation. Cholesterol (7) was determined on all sera and triglyceride (8) on 71. Some of the sera were also studied by starch-gel electrophoresis to determine whether this method would provide information not obtained by the other techniques. When atypical patterns were obtained on the human sera by paper electrophoresis or ultracentrifugation, they were also studied by starch-gel electrophoresis. The sera of 24 pigeons, 12 of the Show-Racer strain (atherosclerosis resistant) and 12 of the White Carneau strain (atherosclerosis prone) were studied individually by paper and by starch-gel electrophoresis and a representative pool of sera of each strain was subjected to ultracentrifugation.

RESULTS AND DISCUSSION

Paper Electrophoresis and Ultracentrifugal Studies

In a preliminary survey of serum lipoproteins of normal and abnormal human beings by the paper electrophoresis techniques we concluded, in agreement with Fredrickson and

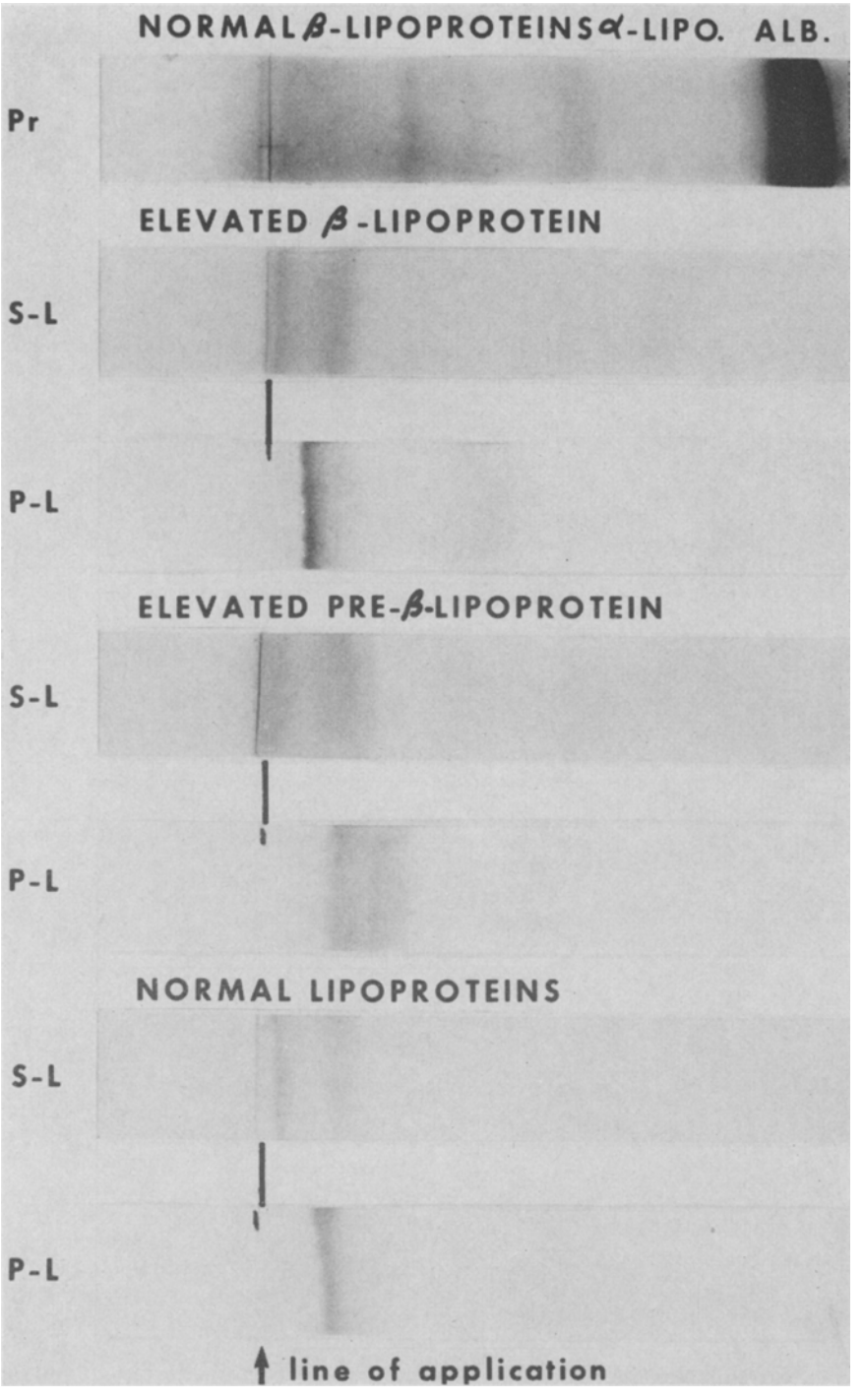

FIG. 1. Thin-layer starch-gel (S) and paper (P) electrophoresis patterns of human serum proteins (Pr) and lipoproteins (L). Amidoschwarz stain.

Lees (9), that while the method is not quantitative, it was capable of demonstrating abnormal distribution of lipoproteins. As a means of reporting the results to the physician the frequently used 0, ±, 1+ to 4+ terminology was used rather than reporting per-

TABLE I

Comparison of Paper Electrophoretic and Ultracentrifugal Patterns of Serum Lipoproteins of the Normal Adult Human Being

Paper electrophoretic pattern of lipoproteins				
	chylomicron	β-lipoprotein	pre-β-lipoprotein	α-lipoprotein
Normal range	0 to ±	2+ to 3+	0 to ±	2+ to 3+
Ultracentrifugal lipoprotein pattern d 1.21				
	chylomicron		mg/100 ml serum	
	-S > 400	-S 25–70	-S 70–400	-S 0–10
Normal adult[a] Human being Range of values	0 to ±	225–390	20–70	180–380

[a]Ranges given do not give variations due to age and sex. They are range of values obtained on samples, on which both paper and ultracentrifugal analyses were made, from 35 normal adult human beings.

centage of lipid stainable material in the different fractions. Since a constant amount of serum (20λ) was always applied to the paper strip, dye intensities of the different bands indicated the relative concentration of the components and also gave some index of the absolute levels (Fig. 1). By the method of classification adopted, normal levels of the four components are chylomicron 0 to ±, β-lipoprotein 2+ to 3+, pre-β-lipoprotein 0 to ±, α-lipoprotein 2+ to 3+. These levels are compared in Table I with normal values obtained by the ultracentrifugal technique. The results obtained by paper electrophoresis were highly reproducible. When the same sera, whether of low, normal or high lipid content, were studied repeatedly, very similar patterns were obtained. Also when serum of an individual on a constant diet was examined at frequent intervals for a period of weeks the patterns obtained were similar.

In the present study sera with cholesterol levels ranging from 78 to 1150 mg/100 ml were analyzed for lipoproteins. Comparison of the results obtained by paper electrophoresis with those by ultracentrifugation showed that abnormally low levels of β-lipoprotein, (i.e., ± to +) by paper electrophoresis were consistently found when -S 25–70 β-lipoprotein levels were below 170 mg/100 ml (Table II). When the concentration of -S 25–70 lipoprotein was increased above 700 mg/100 ml the β-lipoprotein by paper electrophoresis was always classed 4+. Twenty-seven of 32 sera with 3+, β-lipoprotein levels had -S 25–70 concentrations between 300 and 450

TABLE II

Comparison of Human Serum Lipoprotein Levels Determined by Paper Electrophoresis and by Ultracentrifugation

α-lipoproteins						
Paper electrophoresis	Relative dye intensity	0	+	2+	3+	4+
U.C.[a] -S 0–10	mg/100 ml average	0	117	220	291	392
	mg/100 ml range	0	108–170	120–320	180–460	320–480
	No. of samples	1	11	32	54	11
Pre-β-lipoprotein						
Paper electrophoresis	Relative dye intensity	0 to ±	+	2+	3+	4+
U.C. -S 70–400	mg/100 ml average	25	96	252	386	1400
	mg/100 ml range	0–87	52–260	100–820	192–670	350–2800
	No. of samples	58	17	19	8	7
β-lipoprotein						
Paper electrophoresis	Relative dye intensity	±	+	2+	3+	4+
U.C. -S 25–70	mg/100 ml average	91	152	253	358	742
	mg/100 ml range	82–100	47–225	160–440	260–700	360–1080
	No. of samples	4	22	41	32	10
Total number of patients' sera = 109						

[a]U.C. = ultracentrifugation at d 1.21.

mg/100 ml, the other five samples had levels between 500 and 700 mg/100 ml. The 58 sera with pre-β-lipoprotein bands classed ± had –S 70–400 levels of less than 87 mg/100 ml, while all (19) classed 2+ or greater, had –S 70–400 levels greater than 100 mg/100 ml. The 17 sera with 1+ pre-β-lipoprotein levels had –S 70–400 levels ranging from 52 to 260 mg/100 ml.

All (12) α–lipoprotein levels classed ± by the paper electrophoretic technique had –S 0–10 lipoprotein levels less than 170 mg/100 ml, while those (21) classed 1+ had –S 0–10 levels less than 250 mg, and those classed 3+ or 4+ (41) had –S 0–10 levels greater than 350 mg/100 ml.

The ultracentrifugal technique used does not permit quantitation of chylomicron lipoproteins, but their presence in increased amount can be observed. In the seven sera in which presumably chylomicrons were increased as indicated by –S>400 "+" the chylomicron band at the starting point on the paper electrophoretic pattern was also increased. When the levels, determined by paper electrophoresis, of the two lipoprotein fractions of high triglyceride content, i.e., chylomicron and pre-β-lipoprotein, were added and compared with the triglyceride concentration of the sera, it was found that levels of these fractions were consistently high when triglyceride levels were increased (Table III).

Occasional paper electrophoresis patterns of sera show lipid stainable material of unusual mobility. Such a pattern, repeatedly observed in a patient with diffuse xanthomatosis and multiple myeloma, showed an intensely stained lipid band with mobility of γ_2–globulin. Detailed studies on this interesting case are being reported elsewhere (10). When the serum was analyzed ultracentrifugally a high concentration of –S 25–40 β–lipoprotein was found. On starch-gel the lipid stainable material did not migrate into the gel but remained at the application point. Paper and starch-gel electrophoretic analysis of the lipoprotein concentrate and of the subnatant fraction from the preparative ultracentrifuge tube (density 1.21), showed that the γ_2–globulin–lipoprotein complex had been broken and separation of the two components had occurred during ultracentrifugation at high salt concentration. Paper, starch-gel and ultracentrifugal patterns of the lipoprotein concentrate were typical of those found in hypercholesteremia. Some patterns of sera show very faintly stained fractions of a slightly brownish tinge having electrophoretic mobility of γ_2, or γ_3–globulins. If a paper electrophoresis strip of these sera stained for proteins is examined a fraction of high protein concentration with mobility similar to that of the atypical lipid-stained band is usually demonstrated.

TABLE III

Comparison of Concentration of Serum Triglyceride and of Triglyceride Rich Fractions Resolved by Paper Electrophoresis

Triglyceride mg/100 ml serum	Sum of Pre-β and chylomicron fractions[a]
10–112	0 to 1½+[b]
113–390	1½+ to 2+
391–1000	3+ to 6+
1001–5000	4½+ to 7+

[a]Units based on relative staining intensity of lipoprotein.
[b]Value of ± in this calculation was classed "½+."

While the paper electrophoresis method for estimation of lipoprotein is not truly quantitative, our results show that an abnormal distribution of lipoproteins can be detected and the degree of abnormality indicated is usually in the correct range as judged by comparison of results with the ultracentrifugal method. Abnormal levels of lipoproteins demonstrated by paper electrophoresis are helpful in indicating the type of lipid abnormality and additional studies needed, and they can serve as a guide to the nutritionist in dietary counseling of hyperlipemic patients.

Starch-Gel Electrophoretic Studies

When serum was studied by thin-layer starch-gel electrophoresis, resolution of the lipoproteins was similar to that obtained when the starch-gel block technique was used. The thin-layer method has the advantage that the patterns can be scanned. In starch-gel the α–lipoproteins migrate at a rate slightly less than that of albumin, while the β–lipoproteins have a mobility usually slightly less than that of slow α_2–globulin. The mobility of the α–lipoproteins tended to be somewhat slower in the ultracentrifugally separated concentrate than in whole serum, which may be due to difference in concentration, and possibly the effect of other proteins present in the serum. When a high concentration of pre–β–lipoprotein was found by paper electrophoresis a fraction migrating faster than the β-lipoprotein fraction was sometimes demonstrated by starch-gel (bottom pattern, Fig. 2). In most cases a fraction or fractions with mobility less than that of the major β–lipoprotein was resolved, and occasionally a single fraction

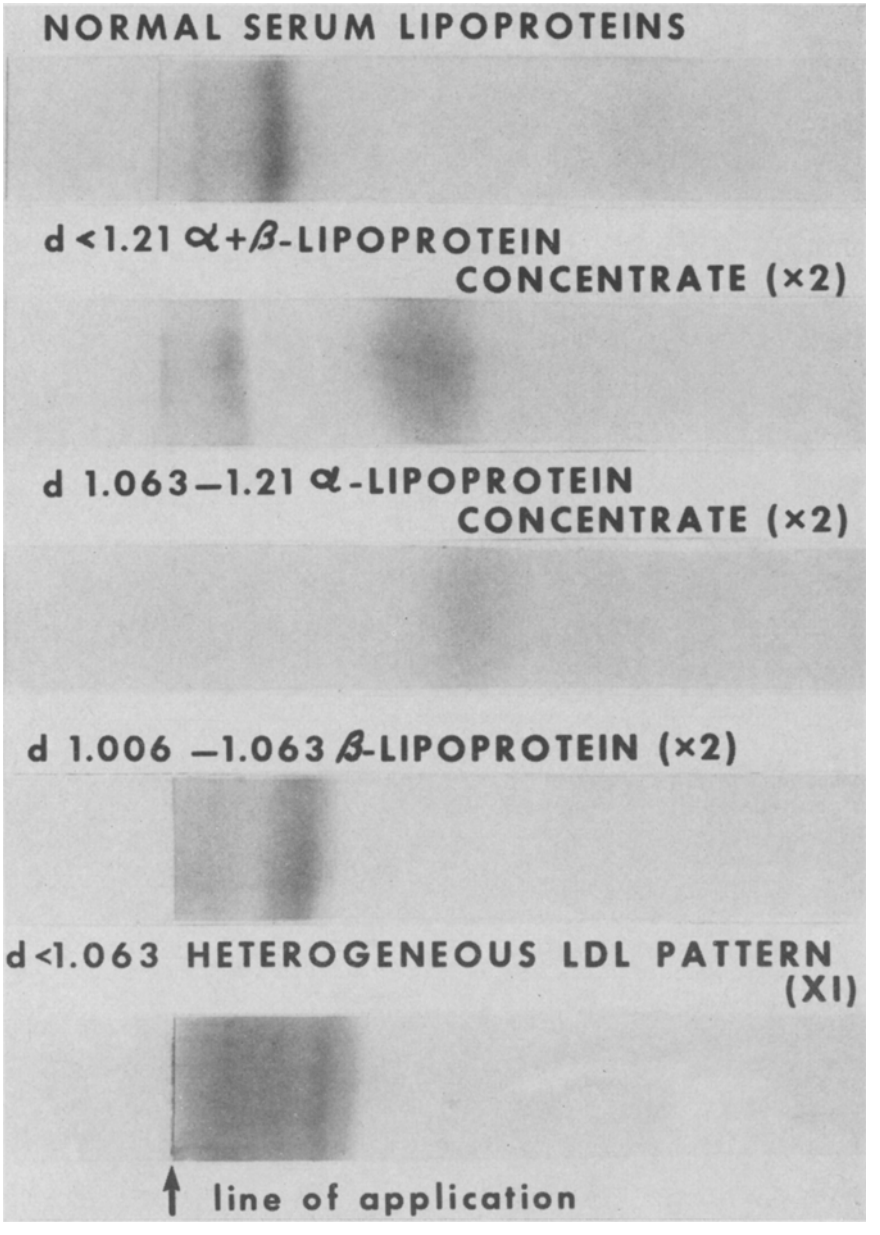

FIG. 2. Thin-layer starch-gel electrophoresis patterns of human serum lipoproteins and ultracentrifugally concentrated lipoprotein fractions. Oil red O stain. Top four patterns of normal human serum. 1. whole serum; 2. d 1.21 α + β lipoprotein concentrate ($\times$2); 3. d 1.063-1.21, α-lipoprotein concentrate (+2); 4. d 1.006-1.063, β-lipoprotein concentrate ($\times$2). Bottom pattern of d $<$ 1.063 lipoprotein fraction from hyperlipemic human serum, which showed by paper electrophoresis increased concentration of chylomicron, and broad intensely stained band with mobility of fast β-lipoprotein, and poorly resolved pre-β-lipoprotein band.

with mobility in β-lipoprotein range was found. When the latter sera were studied by two-dimensional electrophoresis, i.e., the first dimension on paper, and then the paper strip after electrophoresis placed on starch-gel and electrophoresis carried out perpendicular to the direction of the first migration, both β– and pre–β–fractions migrated from the paper

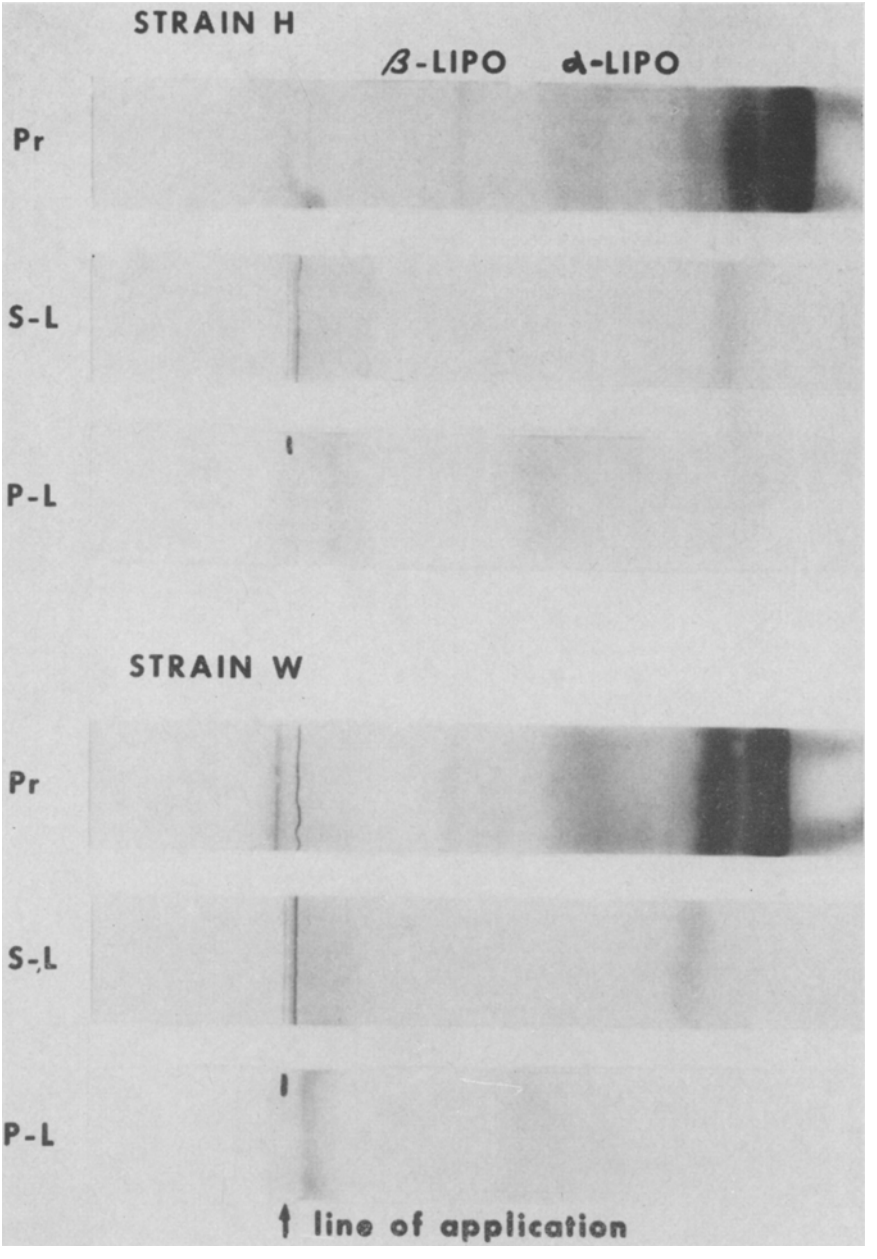

FIG. 3. Thin-layer starch-gel (S) and paper (P) electrophoresis patterns of pigeons' serum lipoprotein (L) and proteins (Pr). Strain H, Show-Racer; Strain W, White Carneau.

into the starch-gel at similar rates. The bands were diffuse and less clearly resolved than usual. In the ultracentrifugal analysis increased levels of –S 70–400 lipoprotein were found in these sera. For use as a method of screening of human sera for lipoprotein abnormalities results by the starch-gel method are not always comparable to those of the other two methods evaluated. The discrepancies are not presently understood. It is possible that an occasional sera in which a component of mobility on starch-gel less than that of the major *β–lipoprotein*, and having a mobility of pre–*β*– by paper electrophoresis are examples of the double *β*–lipoprotein: a new genetic variant in man described by

Seegers et al. (11). It is more likely, however, that the pore size of the starch-gel is small enough to retard the migration of part of the pre–β–lipoprotein. Further investigation of the lipid and protein components of these sera may explain the lack of agreement.

The starch-gel technique has been useful in more precise characterization of the lipoproteins in ultracentrifugal concentrates of serum lipoproteins which by paper or ultracentrifugal techniques showed atypical properties. One such concentrate when subjected to electrophoresis on starch-gel and stained for protein was found to have an intensely stained band, not normally present, of mobility faster than that of β–lipoprotein. The band was identified by immunoelectrophoresis as γ-A globulin (12).

Examination of the lipoproteins of sera of pigeons of both the Show-Racer and White Carneau strains by paper and by starch-gel electrophoresis showed that α- and β-lipoprotein fractions were clearly separated by both methods. While two α-lipoprotein fractions were resolved by paper electrophoresis at least four clearly resolved bands with mobilities in the range of α-lipoproteins were demonstrated on starch-gel (Fig. 3). By ultracentrifugation lipoprotein components with flotation rate between –S 0-15 and –S 20-70 were demonstrated. For sera of this particular species starch-gel electrophoresis gave greater resolution of lipoproteins than was obtained by the other two methods. The small pore size of the starch-gel probably was a factor in accomplishing the resolution of α-lipoprotein subfractions. Further studies of pigeon sera to demonstrate possible differences between the two strains are in progress.

ACKNOWLEDGMENTS

This work was supported in part by Grant HE-6835 from the National Heart Institute. Technical assistance by S. Barany, J. Paksi and Mrs. K. Rash. The pigeon sera were provided by S. C. Smith, University of New Hampshire.

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[Received April 24, 1968]

Labeling of Liver and Plasma Lecithins After Injection of 1-2-^{14}C-2-Dimethylaminoethanol and ^{14}C-L-Methionine-Methyl to Choline Deficient Rats

B. LOMBARDI, P. PANI, F. F. SCHLUNK, and **CHEN SHI-HUA,** Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213

ABSTRACT

Groups of rats were fed a choline-deficient (CD) or a choline-supplemented (CS) diet for 15 hr. Labeling of liver and plasma cephalins and lecithins was followed with time after injection of ^{14}C-L-methionine-methyl or 1-2-^{14}C-2-dimethylaminoethanol, either alone or together with ^{3}H-S-adenosyl-L-methionine-methyl. A reduced concentration of liver and plasma lecithins was found in CD rats. In the same animals, labeling of the phospholipid fractions was considerably greater and faster than in CS rats. Administration of choline to rats previously fed the CD diet resulted in both an increased concentration of liver and plasma lecithins and a reduction in the labeling of liver and plasma lecithins to levels seen in control rats. These results suggest that in CD rats, while the overall synthesis of lecithins may be reduced due to insufficient availability of choline, the synthesis of lecithins via stepwise methylation of cephalins may be increased.

INTRODUCTION

The effects of choline deficiency (CD) on rat liver have long been suspected to stem from an impaired synthesis of lecithins (1). This notion, however, has lately been questioned or discounted (2,3) because of the difficulty in reconciling two sets of apparently contradictory observations. On one hand, it has been found that a reduction in the level of liver (4,5) and plasma (4-7) phospholipids occurs within 24 hr of choline deficiency in the rat, and that such a reduction accompanies the development of the hepatic lesions (4). On the other hand, evidence has been obtained, from ^{32}P incorporation studies, that the turnover of liver lecithins is increased in choline deficient animals (8, 9). A plausible explanation of these observations may have been provided by recently acquired knowledge about the biosynthesis and heterogeneity of liver and plasma lecithins. It has been well established in recent years that lecithins are a very heterogeneous class of lipids, and several subclasses or species have been separated and identified on the basis of their fatty acid composition (10). It has also been shown that the biosynthesis of lecithins occurs via several pathways, and this multiplicity has been considered as largely responsible for the heterogeneity of these phospholipids (11). Thus, it seems possible that in CD rats, synthesis of only some species of lecithins may be increased, accounting for the increased ^{32}P turnover, while the overall synthesis of lecithins may be decreased, accounting for the lower levels in liver and plasma. This possibility is now experimentally testable and we have, therefore, undertaken to do so.

Of the various pathways, two are of quantitative importance for net synthesis of lecithins in the liver (12): incorporation of preformed, free choline via cytidine diphosphate choline and diglycerides (direct pathway) and the stepwise methylation of phosphatidylethanolamines by S-adenosylmethionine (indirect pathway). There is evidence that in rats, especially males (13), the direct pathway is the most active. In this paper we report the results of experiments mainly designed to obtain kinetic data on the incorporation of some labeled precursors for the synthesis of lecithins via the indirect pathway. After injection of 2-dimethylaminoethanol or methionine, or both, a marked difference between choline-supplemented (CS) and CD rats was observed in the labeling of liver and plasma lecithins.

MATERIALS AND METHODS

General Procedures

Male rats of the Sprague-Dawley strain (Sprague-Dawley, Co., Madison, Wisconsin) weighing approximately 100 g were used. Preparation of the animals for the experiments, preparation of the CS and of the CD diets (14), and measurement of diet intake were performed as previously reported (4,7). Labeled precursors were injected after 15 hr of feeding the experimental diets, with the animals

having access to the diets up to the time of killing. Rats fed the CD diet represented the experimental animals, and those fed the CS diet the controls.

Administration of Radioactive Compounds

Radioactive compounds were dissolved in saline (0.9% NaCl) and 0.2 ml of solution was injected into a saphenous vein under light ether anesthesia. The following were used: 1-2-^{14}C-2-dimethylaminoethanol (0.60 and 1.52 mc/mM, Tracerlab, Waltham, Massachusetts), ^{14}C-L-methionine-methyl (13.4 mc/mM, New England Nuclear Corp., Boston, Massachusetts) and ^{3}H-S-adenosyl-L-methionine-methyl (1050 mc/mM, Tracerlab).

Collection of Tissue Samples.

Groups of animals were sacrificed at various time intervals after the injection of the labels, as indicated under the Results section. Five minutes before killing, 5 mg of pentobarbital per 100 g of body weight was injected intraperitoneally. Blood and liver samples were obtained, stored and prepared for analysis as previously reported (4,7).

Analytical Procedures

Plasma and liver total lipids were extracted (15) with a mixture of chloroform-methanol (2:1, v/v) and separated into neutral lipids and phospholipids by chromatography on silicic acid columns (16). Triglycerides (16), lecithins and cephalins (17) were isolated by TLC on silica gels. Triglycerides were determined (18) after elution from the silica gel with chloroform. The phospholipid fractions were eluted with a mixture of chlorofrom-methanol-ammonia (1:3:1, v/v). One aliquot of the eluates was used for determination of lipid P (19); a factor of 25 was used to convert weight of lipid P to weight of lecithins and cephalins. A second aliquot was pipetted into counting vials, dried under nitrogen, and then dissolved in 0.5 ml of methanol followed by 15 ml of a toluene scintillation mixture. Aliquots (0.2 ml) of the radioactive solutions used for injection into the animals were brought to 250 ml with methanol, and 0.5 ml immediately taken and similarly prepared for counting. Radioactivity was measured in a Packard liquid scintillation spectrometer (Model 3002) set for single or double (20) label counting. Specific and total activities were calculated as previously reported (5). Differences between the means were checked with student t-test and regarded to be significant if $P \leq 0.05$. Fatty acid methyl-esters were prepared and purified by the method of Marinetti (21). They were analyzed with an FM model 400 gas chromatograph, which was equipped with a hydrogen flame detector and a ⅛ in. I.D. by 6 ft column packed with 15% ethylene-glycol-succinate on 80-100 mesh gas-chrom P. The column temperature was 160 C, and the flow rate of the carrier gas (argon) was approximately 100 ml/min. The paper cutting and weighing method was used to calculate the composition of the mixtures. Quantitative results with fatty acid standards (F-N.I.H.) agreed with the stated composition data with a relative error of less than 5%. Hydroquinone was added to the solvents used in the extraction and elution (5 mg/100 ml), TLC (50 mg/100 ml) of the lipids, and the preparation and purification of the methyl-esters (5 mg/100 ml).

RESULTS

Three experiments were performed in rats which had been fed the diets for 15-19 hr. As can be seen from Table I, the livers of rats fed

TABLE I

Liver and Plasma Lipids in Rats Fed a Choline-Deficient (CD) or a Choline-Supplemented (CS) Diet for 15-19 hr[a]

Exp.	Diet (No. of rats)	wt. g	Liver mg/100 g body weight TG[b]	Liver mg/100 g body weight PC[b]	Liver mg/100 g body weight PE[b]	Plasma mg/100 ml PC[b]	Plasma mg/100 ml PE[b]
1	CS (12)	6.39 ± 0.24	33.9 ± 6.6	99.8 ± 12.8	42.1 ± 1.4	99.3 ± 6.4	4.4 ± 0.5
	CD (12)	6.45 ± 0.03	120.6 ± 4.9[c]	68.5 ± 7.8[c]	48.2 ± 3.8	68.1 ± 3.5[c]	3.4 ± 0.3
2	CS (12)	6.12 ± 0.12	33.4 ± 2.2	65.5 ± 2.2	43.7 ± 1.3	74.9 ± 5.7	2.7 ± 0.3
	CD (12)	6.29 ± 0.12	120.8 ± 9.8[c]	43.6 ± 1.2[c]	45.5 ± 0.7	61.2 ± 2.4[c]	2.0 ± 0.1
3	CD (5)	5.48 ± 0.13	136.9 ± 16.7	43.8 ± 1.6	42.8 ± 2.1	64.3 ± 3.3	4.3 ± 0.4
	CS (5)	5.49 ± 0.25	28.4 ± 6.2[c]	62.9 ± 1.9[c]	42.2 ± 0.7	83.9 ± 2.1[c]	5.5 ± 0.5
	CD & Ch (5)[d]	5.56 ± 0.10	50.3 ± 7.0	65.9 ± 1.9	46.1 ± 0.9	79.0 ± 3.3	5.01 ± 0.4

[a] Each value represents the mean ± SEM.

[b] TG, triglycerides; PC, lecithins; PE, cephalins.

[c] $P < 0.05$.

[d] Rats fed the CD diet and given choline orally after 9 and 12 hr.

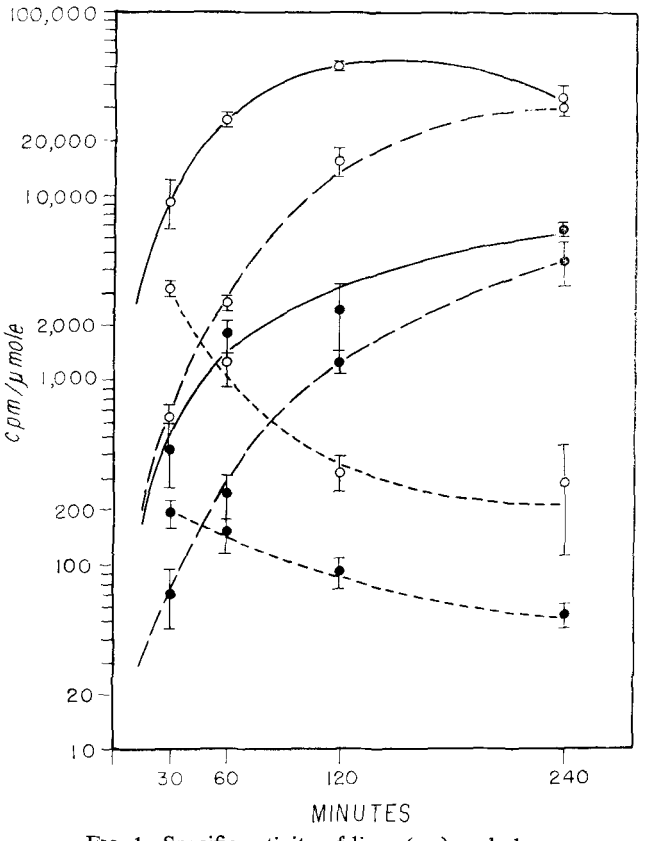

FIG. 1. Specific activity of liver (—) and plasma (— — —) lecithins and of liver cephalins (- - -) after injection of 1-2-^{14}C 2-dimethylaminoethanol to rats fed a choline supplemented (●) or a choline deficient (○) diet (exp. 1). Each point represents the mean ± SEM of 3 rats.

the CD diet contained from 3.5 to 4.8 times as much triglycerides as did the livers of animals fed the CS diet. This increase was accompanied by significantly lower concentrations of both liver and plasma lecithins. In experiment 1, the concentrations of liver and plasma lecithins were higher, in both CS and CD rats, than those seen in experiments 2 and 3. The reason for this finding is not readily apparent; it may however reside in seasonal variations or in the fact that different batches of animals were used in the experiments. In rats fed the CD diet, but given choline orally after 9 and 12 hr (exp. 3), the concentration of liver and plasma lecithins was quite similar to that in CS animals.

Labeling of Lecithins by 1-2- ^{14}C-2-Dimethylaminoethanol (Exp. 1)

It has been shown that rat liver incorporates 2-dimethylaminoethanol into phosphatidyldimethylaminoethanol, a cephalin (22,23). This cephalin is one step removed from lecithins since its methylation by S-adenosylmethionine yields lecithins. Thus, the incorporation of 2-dimethylaminoethanol into lecithins is essentially a 2 step reaction: synthesis of the cephalin, either via the cytidine pathway (24) or through an exchange reaction (25), and methylation of the cephalin to lecithin.

1-2-^{14}C-2-Dimethylaminoethanol (8 μc/rat) was therefore injected into CS and CD rats, and groups of animals were killed 30, 60, 120 and 240 min thereafter. The specific activity (SA) and the total activity (TA) of liver and plasma cephalins[1] and lecithins were determined.

The SA time curves (Fig. 1) revealed a

[1] This fraction is presumably a mixture of phosphatidylethanolamine and its mono- and dimethyl-derivatives. Resolution of the cephalins was not attempted in this study. Therefore, the values for the radioactivity of the cephalins are not accurate. They are reported here only to give an indication of the relationship of their time-curves to those of lecithins.

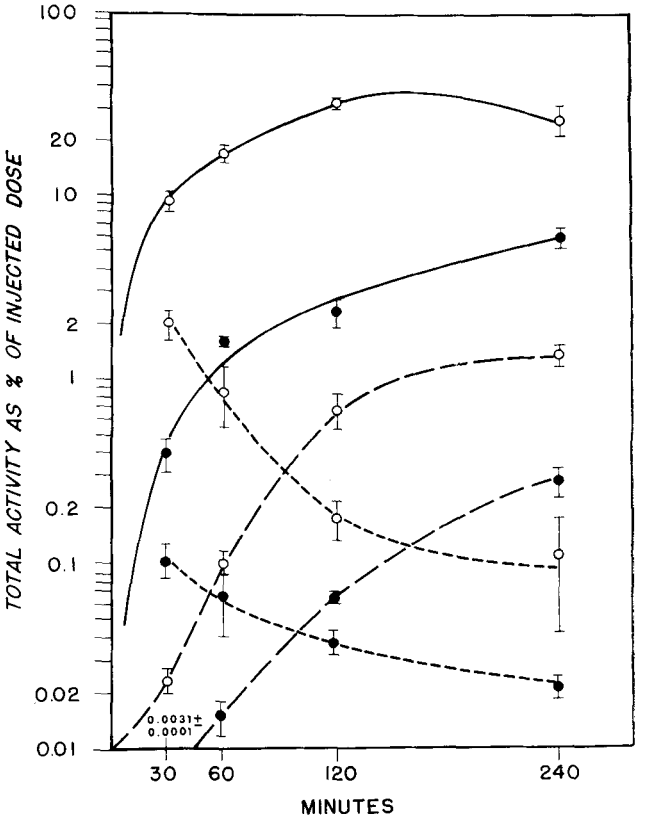

FIG. 2. Total activity of liver (—) and plasma (— — —) lecithins and of liver cephalins (- - -) after injection of 1-2-^{14}C 2-dimethylaminoethanol to rats fed a choline supplemented (●) or a choline deficient (○) diet (exp. 1). Each point represents the mean ± SEM of 3 rats. At the 30 min point, the value of plasma lecithins in choline-supplemented rats was 0.0031 ± 0.0001.

striking difference between CD and CS rats. Labeling of liver cephalins and of liver and plasma lecithins was both considerably greater and faster in experimental animals than in controls. In both CD and CS rats the label appeared to enter in, and disappear from, the pool of liver cephalins quite rapidly. Accompanying the decrease in the SA of liver cephalins was a rise in the SA of liver lecithins which was higher than that of cephalins within 30 min and reached a maximum of 50,314 cpm at 120 min in CD rats, and of 7,061 cpm at 240 min in controls. The SA of plasma lecithins rose also steadily with time, but initially the rise lagged behind that of the SA of liver lecithins. There was no significant difference between CD and CS rats in the SA of plasma cephalins (not shown in Fig. 1). In both experimental and control animals it rose with time to a maximum at 120 min and then declined. The values (mean ± standard error of the mean) for the SA of plasma cephalins in CD and in CS rats at the 30, 60, 120 and 240 min points were respectively: 204 ± 17 vs. 203 ± 43; 536 ± 63 vs. 570 ± 14; 641 ± 148 vs. 832 ± 396; and 162 ± 15 vs. 180 ± 70.

Total radioactivities are plotted in Figure 2. It can be seen from this figure that the fraction of the injected ^{14}C incorporated into liver and plasma lecithins was considerably and consistently larger in CD rats than in controls. It appears, therefore, that the higher SA observed in CD rats was not simply due to the smaller pool of liver and plasma lecithins in these animals.

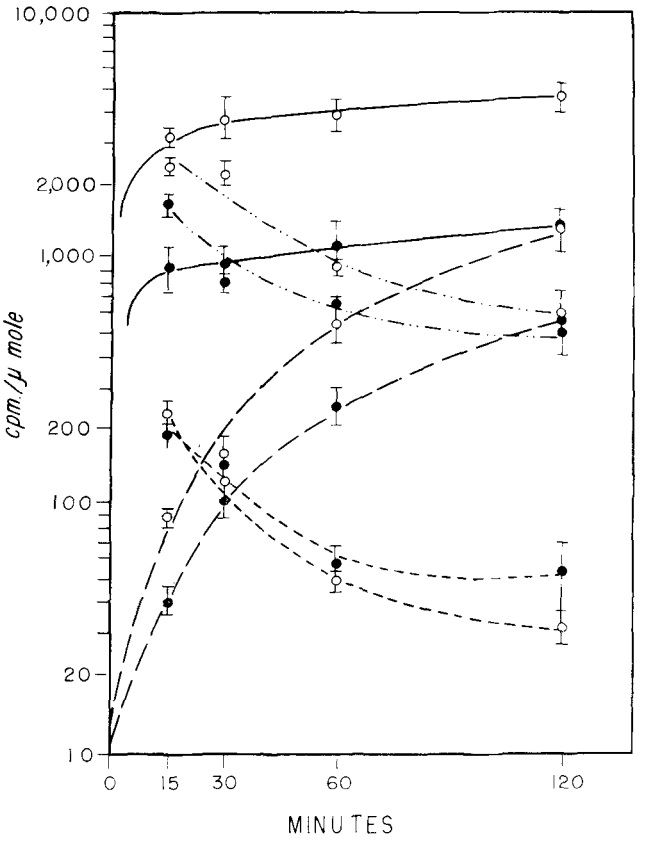

FIG. 3. Specific activity of liver (—) and plasma (— — —) lecithins and of liver (- - -) and plasma (— • • —) cephalins after injection of ^{14}C L-methionine-methyl to rats fed a choline supplemented (●) or a choline deficient (○) diet (exp. 2). Each point represents the mean ± SEM of 3 rats.

Labeling of Lecithins by ^{14}C-L-Methionine-Methyl (Exp. 2)

Another way of testing the indirect pathway for lecithin synthesis is that of determining the incorporation of the methyl group of methionine, that is, methylation of cephalins to lecithins.

^{14}C-L-Methionine-methyl (8 μc/rat) was therefore injected into CS and CD rats and groups of animals were killed 15, 30, 60 and 120 min thereafter. The SA and TA of liver and plasma cephalins and lecithins were determined.

The SA time curves are shown in Figure 3. Again, the SA of liver and plasma lecithins was at all points significantly higher in CD rats than in controls. However, the difference was not as great as that seen after injection of 1-2-^{14}C-2-dimethylaminoethanol. This smaller difference could very well be the result of the fact that incorporation of the methyl group of methionine into liver lecithins, and probably also liver cephalins, was considerably faster than that of dimethylaminoethanol in both CD and CS rats. Indeed, there was very little increase in the SA of liver lecithins after 15 min. Also the SA of liver cephalins was considerably lower than that of plasma cephalins in contrast with the findings in the previous experiment. Furthermore, no difference was seen between CD and CS rats in the SA of liver cephalins while the SA of plasma cephalins was somewhat higher in experimental than in control rats. The above difference also seems to indicate that in CD rats it is the synthesis of phosphatidyl-dimethylaminoethanol from the free base which is stimulated, rather than methylation of cephalins to lecithins.

Total radioactivity recovered at the various time intervals in liver cephalins and in liver

TABLE II

Specific and Total Activity of Liver and Plasma Phospholipids 60 min After the Injection of 1-2-^{14}C-2-Dimethylaminoethanol and ^{3}H-S-Adenosyl-L-Methionine-Methyl to Rats Fed a Choline-supplemented (CS) or a Choline-deficient (CD) Diet (Exp. 3)[a]

Tissue	Diet	Specific Activity DPM/μ mole $\times 10^{-3}$				Total Activity DPM/10^{-3}[c]			
		PC[b]		PE[b]		PC[b]		PE[b]	
		C^{14}	H^{3}	C^{14}	H^{3}	C^{14}	H^{3}	C^{14}	H^{3}
Liver	CD	24.25 ± 2.83	12.65 ± 1.92	4.12 ± 0.90	2.22 ± 0.46	1377 ± 183 (10.97)	633 ± 66 (1.59)	231 ± 57 (1.84)	124 ± 29 (0.31)
	CS	1.51 ± 0.33[d]	2.41 ± 0.46[d]	0.58 ± 0.24[d]	0.59 ± 0.12[d]	125 ± 30[d] (1.00)	198 ± 40[d] (0.50)	53 ± 11[d] (0.43)	32 ± 7[d] (0.08)
	CD & Ch[e]	2.09 ± 0.54	1.90 ± 0.32	0.37 ± 0.16	0.58 ± 0.18	173 ± 4 (1.38)	158 ± 23 (0.40)	59 ± 9 (0.47)	34 ± 11 (0.086)
Plasma	CD	1.99 ± 0.30	3.30 ± 0.49	3.47 ± 1.24	106 ± 13	9.20 ± 1.57 (0.073)	15.21 ± 2.31 (0.038)	0.99 ± 0.29 (0.008)	36.79 ± 7.20 (0.092)
	CS	0.15 ± 0.02[d]	0.81 ± 0.15[d]	0.31 ± 0.07[d]	92 ± 25	0.91 ± 0.31[d] (0.007)	4.78 ± 0.92[d] (0.012)	0.12 ± 0.03[d] (0.001)	32.61 ± 7.19 (0.082)
	CD & Ch[e]	0.18 ± 0.05	0.80 ± 0.06	0.44 ± 0.12	90 ± 15	0.95 ± 0.24 (0.0075)	4.38 ± 0.26 (0.011)	0.15 ± 0.04 (0.0012)	30.69 ± 4.58 (0.077)

[a] Each value represents the mean ± SEM of 5 rats.

[b] PC, lecithins; PE, cephalins.

[c] Values in parenthesis: percentage of injected ^{14}C or ^{3}H.

[d] $P < 0.05$.

[e] Rats fed the CD diet and given choline orally after 9 and 12 hr.

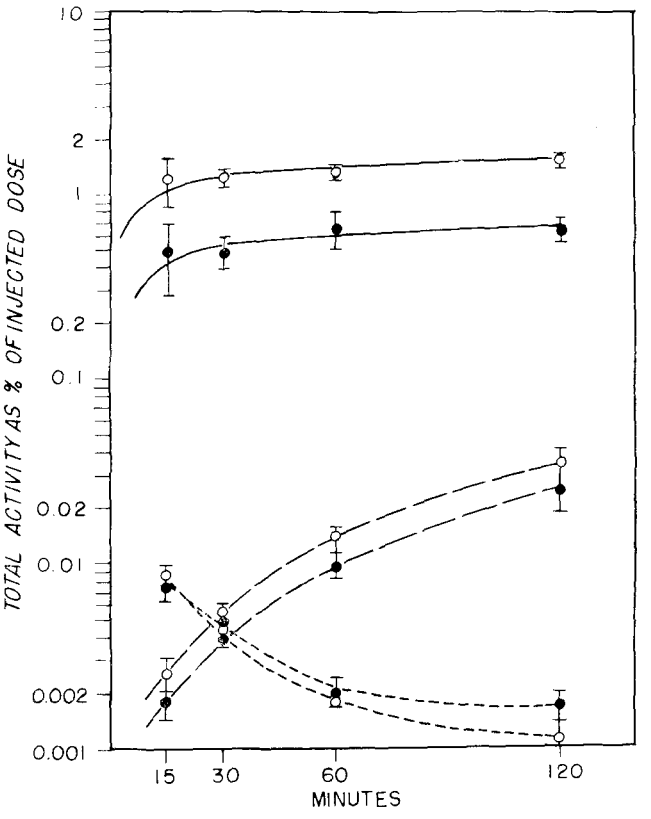

FIG. 4. Total activity of liver (—) and plasma (— — —) lecithins and of liver cephalins (- - -) after injection of ^{14}C L-methionine-methyl to rats fed a choline supplemented (●) or a choline deficient (○) diet (exp. 2). Each point represents the mean ± SEM of 3 rats.

and plasma lecithins is plotted in Figure 4. There was no substantial difference between CD and CS rats in the fraction of the injected dose incorporated into liver cephalins and plasma lecithins. However, a significantly larger fraction was incorporated into liver lecithins of experimental rats.

Effect of Choline on the Labeling of Lecithins by 1-2 ^{14}C-2-Dimethyl-Aminoethanol and ^{3}H-S-Adenosyl-Methionine-Methyl (Exp. 3)

It was previously shown that over 60% of the daily intake of either diet is consumed by the rats during the first 6 hr (4). Thus, to relate the above described changes between CD and CS rats to the lack of choline in the diet, the following experiment was performed. Groups of rats were fed the CD or the CS diet. After 9 hr, and again after 12 hr, 30 mg of choline in 0.5 ml of saline was administered by stomach tube to half of the CD rats; saline (0.5 ml) was similarly administered to the remaining CD rats and to the CS animals. After the rats had been on the diets for 15 hr, 7 μc of 1-2-^{14}C-2-dimethylaminoethanol and 15 μc of ^{3}H-S-adenosyl-L-methionine-methyl in 0.2 ml of saline were injected into each rat, and the animals were killed 60 min thereafter. The two precursors were chosen for the same reasons as before; however, the use of the two labels allowed to perform a single experiment. The ^{14}C and ^{3}H specific and total activities of liver and plasma cephalins and lecithins were determined. The results are presented in Table II.

In CD rats given saline orally, labeling of liver cephalins and of liver and plasma lecithins was again greater than in CS rats. Administration of choline to rats fed the CD diet completely obliterated the difference. Indeed in these animals not only the SA and TA, but also the

TABLE III
Fatty Acid Composition of Liver Lecithins of Rats Fed a Choline-supplemented (CS) or a Choline-deficient (CD) Diet[a]

Diet	Fatty Acids (Wt. %)					
	16:0	18:0	18:1	18:2	20:4	22:6
CS	28.6 ± 1.2	14.3 ± 0.9	15.6 ± 0.4	17.2 ± 0.6	17.2 ± 0.7	4.1 ± 0.3
CD	25.6 ± 0.4[b]	16.0 ± 0.4	13.1 ± 0.5[b]	12.3 ± 0.5[b]	25.0 ± 0.9[b]	4.0 ± 0.5

[a] Each value represents the mean ± SEM of 7 rats.
[b] $P < 0.05$.

concentration (Table I) of the phospholipid fractions were quite similar to those in rats fed the CS diet.

In this experiment, the ^{3}H-SA of plasma cephalins was much higher than that of any other fraction. The reason for this finding is not readily apparent. It should be noted, however, that some of the data on plasma cephalins may not be very accurate since the determinations were performed on very small amounts of this phospholipid (0.02-0.03 μmole).

In contrast to the finding in experiment 2 not only the ^{3}H-SA, but also the fraction of injected ^{3}H incorporated into plasma lecithins was significantly higher in CD than in CS rats.

Fatty Acid Composition of Liver Lecithins

The fatty acid composition of liver lecithins was determined in two groups of seven rats fed either the choline-supplemented or the choline-deficient diet for 15 hr.

As can be seen from Table III, liver lecithins of CD rats contained significantly more arachidonic acid and less linoleic acid than lecithins of the CS group. The latter contained also slightly more palmitic and oleic acid and less stearic acid.

DISCUSSION

Labeling of liver and plasma cephalins and lecithins occurred in both CS and CD rats after injection of ^{14}C-L-methionine-methyl, or of 1-2-^{14}C-2-dimethylaminoethanol either alone or together with ^{3}H-S-adenosyl-L-methionine-methyl. Kinetic data (Fig. 1-4) suggested that the labels were incorporated first, and most rapidly, into liver cephalins, then into liver lecithins, and subsequently into plasma lecithins. These results are in keeping with the notion of the formation of some liver lecithins by stepwise methylation of liver cephalins (11, 12) and the derivation of plasma lecithins from liver lecithins (12). A marked difference was however observed between CD and CS animals since labeling of the phospholipids appeared to be faster, and was greater, in the deficient animals than in the controls.

One of the earliest signs of choline deficiency in the rat is a lowering in the concentration of liver and plasma phospholipids (4-7). The results of Table I show that lecithins partake in such a decrease (as do sphingomyelins and lysolecithins—unpublished results). Evidence has been obtained by Isozaki et al. (26) that administration of choline to CD rats stimulates the synthesis of lecithins via the direct pathway. In experiment 3, in which choline was administered to rats that had ingested the CD diet, the concentration of liver and plasma lecithins rose to the level in controls (fed the CS diet). At the same time, the extent of labeling of liver and plasma lecithins decreased to that seen in the same controls. These results could be interpreted as indicating that in CD rats there is, on the one hand, a decrease in overall synthesis of lecithins due to lack of a substrate, choline, for the major biosynthetic pathway and, on the other, an increased synthesis via the stepwise methylation of cephalins. The increased synthesis via the indirect pathway could then represent an attempt to correct the major defect, and could be a factor in explaining the higher turnover of lecithins observed after ^{32}P-administration (8,9), despite the lower concentration in liver and plasma.

No information was sought in the present experiments about the vascular clearance and liver uptake of the injected labeled compounds, or the pool size and SA of the various intermediate precursors. Whether or to what extent a difference between CD and CS rats in any of these parameters can account for the observed difference in the labeling of lecithins is presently being investigated. Another possibility can be excluded, namely, that the greater labeling of liver lecithins may result from an accumulation of newly synthesized lecithins due to their impaired release into plasma as a moiety of serum lipoproteins (5,7). Indeed a smaller pool of liver lecithins was present in CD animals than in controls, and both the SA and TA of plasma lecithins was greater in CD rats than in controls even though the concentration was lower.

One expected consequence of a shift in the pathways for lecithin synthesis by the liver of CD rats would be a change in the overall fatty acid composition of the phospholipid (11), that is a redistribution of the molecular species of lecithins. That such a redistribution in fact occurred is shown by the results of Table III. It has been suggested by Isozaki et al. (26) that liver lecithins containing highly unsaturated fatty acids and stearic acid are synthesized mainly through the indirect pathway, and those containing less unsaturated fatty acids and palmitic acid via both the direct and indirect pathway. Indeed, it has been shown by Lyman et al. (27, 28) that there is a good correlation between methylation of phosphatidylethanolamines and synthesis of liver lecithins rich in arachidonic and stearic acid. Thus the finding that liver lecithins of CD rats contain relatively more arachidonic and stearic acid and less linoleic, oleic and palmitic acid than the lecithins of controls corroborates the results of the tracer experiments indicating an increase in the synthesis of lecithins via the indirect pathway.

It seems then possible that, at least during the early stages of choline deficiency in the rat, the pathologic effects on the liver may result not merely from an insufficient availability of lecithins, but also from the lack of specific lecithins. These effects could involve the metabolism of either soluble (serum) lipoproteins or cellular membranes (microsomal, Golgi and plasma membranes), or both, thereby accounting for the impaired release into plasma of hepatic TG (5), serum lipoproteins and serum albumin (7). A decreased concentration of lecithins has been found in microsomes isolated from the liver of CD rats (unpublished observations).

The results and considerations presented in this paper are at variance with others in the literature on choline deficiency. However, the wide differences in experimental conditions used in different laboratories makes a direct comparison between them rather difficult. Of the experimental conditions, the age of the rats, the length of feeding and the amounts and types of proteins and fats in the diets may be the most critical.

ACKNOWLEDGMENTS

The valuable assistance of Miss Eileen LaRocca and of Mrs. Gai Barry is acknowledged. This work was supported by research grant AM-06334 from the National Institute of Arthritis and Metabolic Diseases, and a research grant GM-10269 and a training grant GM-135 from the National Institute of General Medical Sciences.

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[Received June 10, 1968]

SHORT COMMUNICATIONS

What is Amaurotic Idiocy?

As RECENTLY AS 1966, amaurotic idiocy was defined as a "neurolipidosis with thesaurization of gangliosides" (1). Extensive discussions evolved about the condition at the symposium on Inborn Disorders of Sphingolipid Metabolism (2). Since lipid chemists occasionally study tissues from patients with that diagnosis, attention is drawn to the fact that amaurotic idiocy comprises several heterogeneous diseases some of which display no detectable abnormalities of the sphingolipid pattern and may not even be associated with a disturbance of lipid metabolism.

Sachs (3) coined the term "amaurotic idiocy" to designate what is now known as Tay-Sachs disease, infantile amaurotic idiocy or, preferably, G_{M_2}-gangliosidosis. Vogt (4) suggested that amaurotic idiocy occurs in several forms all characterized by neuronal lipid accumulation, distinguishable simply by the age of onset. Accordingly, contemporary medical writers recognize a congenital, infantile, late infantile, juvenile and adult form of amaurotic idiocy and thus relegate the original entity defined by the work of Tay and Sachs to a subtype of a seemingly well-defined disease. With the discovery by Klenk (5) of large quantities of a hitherto unknown glycolipid, later identified as ganglioside G_{M_2} in two brains with Tay-Sachs disease, neuropathologists and clinicians drew the conclusion that the other subtypes also harbor increased amounts of gangliosides. This has indeed proven correct for certain cases of late infantile amaurotic idiocy (Bielschowsky-Jansky type) which O'Brien et al. (6) defined as G_{M_1}-gangliosidosis. In other cases of amaurotic idiocy chemical studies did not reveal a significant accumulation of gangliosides. Several unsound hypotheses have been advanced to explain this negative finding.

The electron microscopic examination of brains with Tay-Sachs disease, championed by Terry and Korey (7) opened a seemingly new approach to the understanding of amaurotic idiocy, by investigating the ultrastructural architecture of the neuronal lipid cytosomes. Using such criteria, Gonatas et al. (8) found it possible to differentiate Tay-Sachs disease, G_{M_2}-gangliosidosis, late infantile amaurotic idiocy of the Bielschowsky-Jansky type, a second (new) type of late infantile amaurotic idiocy, and juvenile amaurotic idiocy. Thus, three conditions are distinguished which seem to answer the definition of late infantile amaurotic idiocy and, seemingly, two more types, one with accumulation of ganglioside G_{M_2} and the second one without accumulation of gangliosides, also designated as "myoclonic variant of cerebral lipidosis" were described by Seitelberger et al. (9) (2). These five subtypes of the subtype late infantile amaurotic idiocy confuse not only the lipid chemist but also neuropathologists and neurologists.

Lipid analyses in this laboratory of brain tissue samples from 18 patients suffering from "amaurotic idiocy" produced the following results. All four brains with Tay-Sachs disease or infantile amaurotic idiocy showed massive accumulation of ganglioside G_{M_2}. Among nine cases of late infantile amaurotic idiocy, one brain contained a 75% relative concentration of ganglioside G_{M_1} whereas the remaining had a normal sphingolipid profile. Four patients fulfilled the clinical criteria of juvenile amaurotic idiocy (Spielmeyer-Vogt type) and one of adult amaurotic idiocy (Kufs' disease). All five brains showed essentially normal relative concentrations of sphingolipids. The TLCs were obtained by a qualitative method (10). Quantitative studies on the same material were carried out by Jatzkewitz (München), R. H. McClure (Columbus), and J. S. O'Brien (Los Angeles). Jatzkewitz wrote that his examinations yielded essentially normal values for all classes and types of sphingolipids; from the other two neurochemists we received verbal assurance that no abnormalities were found. These findings are in agreement with the observations of Svennerholm (Göteborg) that cases of juvenile amaurotic idiocy occurring in Sweden never disclosed abnormalities of the sphingolipid pattern. In a recent publication. Duffy et al. (11) described a patient suffering from what appeared to be late infantile amaurotic idiocy. There were changes in the relative proportions of gangliosides "which are considered to be non-specific and commonly seen in a variety of diseases."

These observations make clear that the division of amaurotic idiocy into subtypes is unfounded. As a consequence, the entire classification should be abandoned. We can now distinguish on clinical, pathomorphological and biochemical grounds between G_{M_1}-gangliosidosis and G_{M_2}-gangliosidosis. Available morphologic and biochemical data suggest that the remaining cases may well form an entity or a group of closely related conditions which we

call Batten's disease or syndrome because Batten (12) recognized and described basic differences between this condition and Tay-Sachs disease.

Light and electron microscopic examinations on approximately two dozen cases of Batten's disease, 12 of which were studied in this laboratory, revealed that the intraneuronal lipid accumulates in the form of insoluble fluorescent lipid complexes. Their ultrastructural architecture spanned a wide range and efforts to classify or subclassify the condition by the fine structure of the lipid cytosomes failed as soon as different regions of the brain and different stages of the disease were examined. The lipid bodies share many if not all characteristics with ceroid and with lipofuscin granules, respectively.

The relationship between ceroid and lipofuscin is poorly understood; it has been suggested that ceroid transforms into lipofuscin by progressive polymerization and oxidation (13).

Since both complexes are constituents of normal tissue, the pathogenesis of Batten's disease seems to rest primarily on an abnormal rate of accumulation or clearance of these "wear and tear pigments" rather than on the accumulation of a single lipid.

WOLFGANG ZEMAN
Division of Neuropathology
Indiana University School of Medicine
Indianapolis, Indiana 46202

ACKNOWLEDGMENT

This work was supported by USPHS Grant NB-04607.

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[Received August 3, 1968]

Free Fatty Acid Level in Rat Liver After Ingestion of Polyunsaturated Fatty Acids

FREE FATTY ACIDS IN THE LIVER are either the product of de novo synthesis, chain elongation, the degradation products of neutral or complex lipids, or the physiological intermetabolite carried from the adipose tissue or derived from dietary fats. It is desirable to determine the concentration and the composition of free fatty acids in the liver and other tissues for the detailed study of fatty acid metabolism. particularly since little data have been reported in the literature. It was the purpose of the present investigation to show the changes in free fatty acid concentration in normal and fatty livers after administration of unsaturated fatty acids. Unsaturated fatty acids are oxidized at a high rate when administered in large amounts. As the free fatty acid level is a factor which regulates oxidation in mitochondria, it can be postulated that this level is changed by feeding unsaturated fatty acids. Isotope studies have shown that the intake of free fatty acids into the liver is proportionate to the plasma free fatty acid concentration (1). The plasma free fatty acid level is lowered by high carbohydrate diet (2). No agreement has been reached concerning the plasma free fatty acid concentration following the ingestion of a high fat diet.

A group of male albino rats of the Sprague-Dawley strain were maintained on the ordinary stock diet of Oriental Yeast Co. Another group of rats were fed a choline deficient diet consisting of 10% casein, 10% gelatin, 20% beef fat, 60% sugar added with cellulose powder, vitamin and salt mixtures and cod liver oil. Rats were fed ad lib. for 3 weeks. After 12 hr of fasting, 0.3 ml of fatty acid methyl esters were ingested through a stomach tube. Each group of rats were divided into five subgroups. Subgroup 1 were controls. Animals of subgroup 2 were given methyl oleate, subgroup 3 linoleate, subgroup 4 γ-linolenate, and subgroup 5 arachidonate. All the methyl esters of polyunsaturated fatty acids were more than 99% pure (from Ono Pharmaceutical Co.). Twelve hours after ingestion, rats were killed by exsanguination. Livers were removed and immediately irrigated with saline through the portal vein. Extraction of lipids and phospholipid analysis were carried out as described

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TABLE I

Free Fatty Acids in Rat Liver After Ingestion of Unsaturated Fatty Acid

Methyl esters administered	Stock diet fed rats	Choline deficient diet fed rats
None	0.32±0.04 (4)	0.73±0.08 (7)
Oleate	0.53±0.06 (3)[a]	0.75±0.13 (5)
Linoleate	0.56±0.07 (4)[a]	1.06±0.12 (6)[b]
γ-Linolenate	0.59±0.05 (4)[b]	1.50±0.20 (6)[b]
Arachidonate	0.56±0.06 (3)[a]	0.80±0.12 (4)

The concentration of free fatty acid is expressed as milligram free fatty acid per gram liver. Values are the average ± standard deviation. Numbers in parentheses are the number of rats used.

[a] $0.001 < p < 0.01$.

[b] $p < 0.001$.

by Rouser et al. (3,4). Free fatty acids were determined after separation by TLC by conversion to methyl esters and determination of moles of esters by the hydroxamic acid procedure of Rapport and Alonzo (5).

The free fatty acid level in the liver was elevated after ingestion of unsaturated fatty acids in all of the subgroups maintained on a stock diet (Table I). No significant difference was noticed between these subgroups. The free fatty acid level in the fatty liver was about twice as high as the level in the normal liver. In fatty livers, the ingestion of oleate and arachidonate failed to produce an elevation of the free fatty acid level, while the ingestion of linoleate and γ-linolenate, especially the latter, resulted in a marked increase in free fatty acid in the liver. There were essentially no differences in the extent of fatty liver between these subgroups.

The composition of free fatty acids in fatty livers showed a significant increase of oleic acid (18:1) and decreases of stearic (18:0) and palmitic acid (16:0) after the ingestion of γ-linolenate. Although the ingestion of linoleate and γ-linolenate resulted in increases of the corresponding fatty acids, (18:2) and (18:3), the amount of these acids in the free fatty acid fraction was still minor, compared with three major fatty acids; palmitic, stearic and oleic acids (Table II).

TABLE II

Free Fatty Acids in the Liver of Rats Fed a Choline Deficient Diet

Fatty[a] acid	Methyl esters administered		
	Oleate	Linoleate	γ-Linolenate
14:0	1.8±0.3	1.2±0.2	1.2±0.2
16:0	41.0±1.6	38.0±1.2	34.9±2.5
:1	5.3±0.6	5.2±0.4	4.4±1.0
18:0	13.6±0.6	11.6±2.0	10.9±0.6
:1	32.2±1.9	33.1±1.6	40.0±3.2
:2 (ω-6)	3.3±0.9	7.1±0.6	3.7±1.0
:3 (ω-6)	.....	.....	1.6±0.4
20:4 (ω-6)	2.8±0.2	3.8±0.5	3.3±0.9

Values are the average of 4 rats (± standard deviation).

[a] Carbon chain length : number of double bonds.

TABLE III

Liver Phospholipid Composition of Rats Fed a Choline Deficient Diet After Ingestion of Unsaturated Fatty Acids

Phospholipid	Methyl esters administered			
	None	Oleate	Linoleate	γ-Linolenate
Phosphatidyl choline	48.0±0.6	48.4±0.3	46.4±1.8	46.9±1.2
Phosphatidyl ethanolamine	28.9±0.5	29.5±1.1	29.9±1.1	29.9±0.8
Phosphatidyl inositol	8.1±0.2	7.6±0.3	7.9±1.0	8.0±0.6
Cardiolipin	5.0±0.3	5.2±0.5	6.0±0.4	5.0±0.5
Sphingomyelin	4.6±0.1	4.6±0.4	4.6±0.6	4.6±0.2
Phosphatidyl serine	3.0±0.0	3.1±0.2	3.3±0.0	3.2±0.1
Lysophosphatidyl choline	1.1±0.2	0.7±0.2	0.6±0.4	0.9±0.1

Values are the average of 3 rats (± standard deviation). Values of minor components are excluded from this table. Recovery of phosphorus was 99.7% in average.

There were no significant differences in phospholipid composition between these subgroups, except a slight increase in cardiolipin following the ingestion of linoleate (Table III). There was no increase of lyso-compounds, which could explain the elevation of free fatty acid level.

It is generally accepted that the free fatty acid level in the liver is related to the free fatty acid concentration in blood plasma. However, the plasma free fatty acid concentration was lower in oil fed rats than in the control animal (Table IV).

The elevation of the free fatty acid level in the liver after the ingestion of polyunsaturated fatty acids may be the result of an increase in the binding site (carrier protein) in hepatic cells. The fatty acid composition was much more like that of free fatty acids in plasma rather than that of triglycerides in the

TABLE IV

Free Fatty Acids in Blood Plasma in Rats Fed the Choline Deficient Diet After the Ingestion of Unsaturated Fatty Acids

Methyl esters administered	Plasma free fatty acid concentration (μeq/liter)
None	586 , 560
Oleate	400 , 334
Linoleate	346 , 346
γ-Linolenate	480 , 400
Arachidonate	386 , 414

Free fatty acid concentration was determined in two samples for each subgroup.

liver. Bernhard et al. (6) reported a high rate of oxidation of unsaturated fatty acids, especially γ-linolenic acid. Elevation of the free fatty acid level in the liver is probably related to an enhancement of oxidation of fatty acids. An increase of the lipolytic activity after feeding polyunsaturated fatty acids might cause the elevation of the free fatty acid level, although the increase of this activity has not yet been detected in the liver (7).

AKIRA YAMAMOTO, MASAHIRO ISOZAKI, TAKESHI ISHIBE and MITSUO NISHIKAWA
The Second Department of Internal Medicine
Osaka University Medical School
Fukushima-ku, Osaka, Japan

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[Received August 6, 1968]

The Dienoic Fatty Acids of Human Skin Surface Lipid

DIENOIC FATTY ACIDS have been known to occur in small amounts in adult human skin surface lipid, however, their structures have hitherto been unknown. This study reports their structures.

To obtain sufficient material we prepared 1103.3 mg of crude methyl esters from 1532 mg of a pooled sample of scalp surface lipid obtained from a 27-year-old man by procedures previously described (1). Figure 1 gives details on the chromatographic preparation of the dienes and their analysis. In chromatogram I, unsubstituted methyl esters were separated from more polar material: unesterified fatty acids, oxidized acids, traces of non-saponifiable matter and possibly methyl esters of hydroxy fatty acids. In chromatogram II the purified methyl esters were separated into groups of saturates, monoenes and dienes. Traces of saturates, monoenes and what appeared to be oxidized products overlapped into the diene fraction, and were removed from the dienes by rechromatography on $AgNO_3$-SiO_2 (chromatogram III). Thus, of the total unsubstituted methyl esters, dienes make up 2% to 3%, monoenes 47% to 48%, the remainder consisting of saturates. A portion of the dienes of chromatogram II and selected fractions of chromatogram III were preparatively gas chromatographed and analyzed (Fig. 1), chain lengths determined by analytical GLC and the products of reductive ozonolysis. The total number of C-atoms of the ozonolysis fragments (aldehyde plus aldester) for each methyl ester was 3 C-atoms less than the total chain length of that ester. No effort was made to identify malondialdehyde which would be buried in the solvent (methylene chloride). Absence of infrared absorption at 10.3 μ of the total diene fraction of chromatogram III (KBr pellet technique), indicated that the dienes were all *cis*.

Table I lists the relative amounts of dienes of different chain lengths, and the ozonolysis products yielded by the isomers at each chain length. Also listed are the deduced structures of these isomers, their relative amounts and a possible mode of formation for the major isomer. Note that C_{18} and C_{20} dienes constitute over 93% of the total dienes. The position isomers 18:Δ5,8 and 18:Δ9,12 constitute 81% of the C_{18} dienes. The 18:Δ5,8 isomer emerges prior to linoleate (18:Δ9,12) in both GLC (on DEGS polyester) and in $AgNO_3$-SiO_2 liquid chromatography, nearly complete separation occurring in the latter system. This observation is possible in the C_{18} series only because both position isomers occur in relatively large amounts whereas in all other chain lengths only one isomer predominates, and the difference in the number of C-atoms between the first double bond and the carbonyl group is large for this pair of isomers.

The data of this paper suggest that human skin contains enzymes capable of inserting a second *cis* double bond between the carbonyl group and the first double bond, separated from the latter by a methylene group. For example, 18:Δ5,8, the most abundant diene, could be formed from 18:Δ8, an isomer known to occur in human surface lipid (3). The latter isomer in turn could be formed by extension of 16:Δ6, the most abundant monoene, by 1 C_2 unit at the carbonyl group. Thus, by a combination of C_2 extensions and an additional desaturation, all the major isomers of

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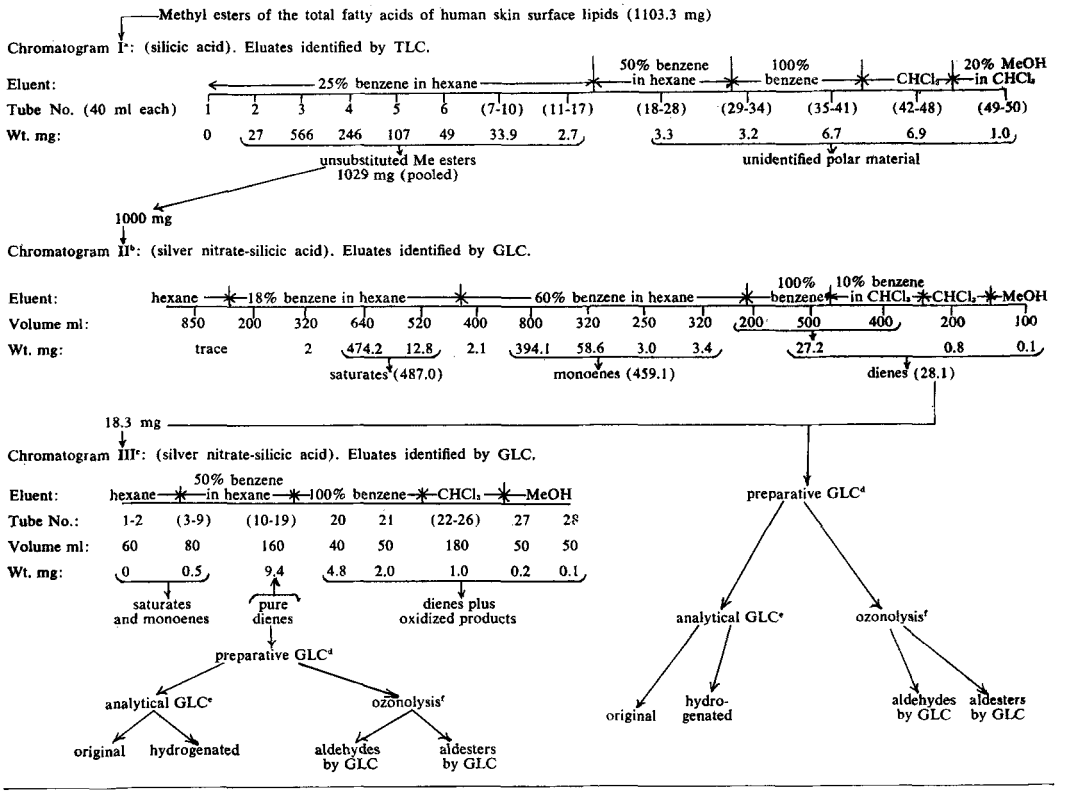
each diene of Table I except linoleic acid could be synthesized from 14:Δ6, 15:Δ6, 16:Δ6, 17:Δ6 and 18:Δ6, all known to occur in human skin surface lipid (3). From these data we cannot tell whether elongation precedes further desaturation or vice versa.

Since a single biosynthetic mechanism can account for the major dienes of each chain length except for linoleic acid, it suggests that the latter has a different origin, perhaps from bacteria or from keratinizing epidermis. Had 12:Δ6 occurred to any appreciable extent it is conceivable that with further elongation and desaturation linoleic acid, an essential fatty acid, could also be synthesized by human skin. Our earlier data (3) suggests that, if present, 12:Δ6 must occur in amounts too small to account for the quantity of linoleic acid seen in this study unless all the 12:Δ6 formed is quantitatively removed by chain elongation to form linoleic acid. If 12:Δ6 does occur, it could also serve as a precursor of the minor components 16:Δ7,10 and 20:Δ11,14.

Structures of the minor components were determined by matching aldehyde and aldester peaks to give dienes of the methylene interrupted type. Although due consideration was given to relative sizes of the aldehyde and aldester peaks, when these substances occurred in small and nearly equal amounts, they could be matched to form structures other than those postulated, for example structures with sep-



[a]Column 2.4 cm ID × 16 cm height packed with 29.8 g silicic acid (Unisil, 100-200 mesh, Clarkson Chemical Co., Inc. Williamsport, Pa.).

[b]Column 4.4 cm ID × 17 cm height packed with 182 g silicic acid impregnated with 25% silver nitrate (CABN, 140-200 mesh, Applied Science Labs, Inc., State College, Pa.).

[c]Column 1 cm ID × 14.2 cm height packed with 7.8 g CABN.

[d]Beckman GC-4 gas chromatograph; 6 ft × ¼ in. OD stainless steel column packed with 8.3 g 3% OV-101 (Applied Science Labs, Inc.) on Gas Chrom Q, 100-200 mesh (Applied Science Labs, Inc.); He flow 100 ml/min; column temp. programed 200-260 C in 32 min; 1/10 effluent to H_2 flame detector.

[e]Instrument as in (d); 10 ft × ⅛ OD stainless steel column packed with 8.2 g 1.5% OV-101 on Chromsorb G, 100-120 mesh, acid washed DMCS treated (Johns Manville Products Corp., Manville, N. J.); He flow 50 ml/min; column temp. 220 C. Fractions also run on polar phase: 15 ft × ⅛ OD stainless steel column packed with 12.9 g 3% stabilized DEGS (Analabs Inc., Hamden 18, Conn.) on Chromosorb G, 100-120 mesh, acid washed DMCS treated (Johns Manville); He flow 50 ml/min; column temp. 180 C.

[f]For ozonolysis procedure see ref. 2 and other refs. there. GLC instrument as in (d); 16 ft × ⅛ OD aluminum column packed with 4.9 g 18% stabilized DEGS on Chromosorb W, 50-80 mesh, acid washed DMCS treated; He flow 50 ml/min; column temperatures 100 C and 130 C.

FIG. 1. Isolation and analysis of the dienoic fatty acids (as methyl esters) of adult human skin surface lipid.

aration of double bonds by more than one methylene group or dienes of the conjugated type, or both. However, evidence to support the postulated structures is that nearly all can be explained by biosynthetic mechanisms involving combinations of elongation and further desaturation of Δ6 and Δ9 monoenes, the latter occurring to a minor extent in human surface lipid.

Preparative GLC revealed small peaks suggestive of dienes with branched chain structures, possibly of the iso and anteiso type. However, we were unable to collect sufficient material to do structure determinations even though we could detect nanogram amounts of aldehyde and aldester after ozonolysis. Thus branched chain dienes, if present, make up a smaller proportion of total dienes than branched chain monoenes do of the total monoenes.

As far as we know, the structures of those dienes derivable from Δ6 monoenes have not been hitherto reported as naturally occurring substances. Since 18:Δ5,8 is the most abundant and characteristic diene to occur in a lipid undoubtedly of sebaceous gland origin, we would like to name it sebaleic acid.

N. NICOLAIDES AND M. N. A. ANSARI
University of Southern California
School of Medicine
Los Angeles, Calif. 90033

ACKNOWLEDGMENT

This work was supported by research grants from the National Institute of Arthritis and Metabolic Diseases, No. AM-10010, U. S. Public Health Service and from the Bireley Foundation, Los Angeles, California.

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[Received August 26, 1968]

TABLE 1

Dienoic Fatty Acids of Adult Human Skin Surface Lipid

Carbon number[a]		Amt. total dienes[d]	Chain length of ozonolysis products		Deduced structure	Isomer distribution	Possible mode of formation of major isomers
Hydrogenated	Original	%	Aldehydes	Aldesters		%	
16	17.32	1.8	8	5	16:Δ5,8	88	14:Δ6 + C_2→16:Δ8→16:Δ5,8
			7	6	16:Δ6,9	7	
			6	7	16:Δ7,10	5	
17	18.30	1.6	10	4	17:Δ4,7	10	
			9	5	17:Δ5,8	59	15:Δ6 + C_2→17:Δ8→17:Δ5,8
			8	6	17:Δ6,9	12	
			7	7	17:Δ7,10	8	
			6	8	17:Δ8,11	8	
			5	9	17:Δ9,12	3	
18	18.95[b]	70.5	10	5	18:Δ5,8	55	16:Δ6 + C_2→18:Δ8→18:Δ5,8
			9	6	18:Δ6,9	5	
			8	7	18:Δ7,10	2	
			7	8	18:Δ8,11	4	
	19.23[c]		6	9	18:Δ9,12	26	(see text)
			5	10	18:Δ10,13	8	
19	20.05	1.8	11	5	19:Δ5,8	86	17:Δ6 + C_2→19:Δ8→19:Δ5,8
			10	6	19:Δ6,9	5	
			9	7	19:Δ7,10	5	
			8	8	19:Δ8,11	3	
			7	9	19:Δ9,12	1	
20	21.02	22.6	13	4	20:Δ4,7	1	
			12	5	20:Δ5,8	2	
			11	6	20:Δ6,9	1	
			10	7	20:Δ7,10	78	16:Δ6 + 2C_2→20:Δ10→20:Δ7,10
			9	8	20:Δ8,11	5	
			8	9	20:Δ9,12	4	
			7	10	20:Δ10,13	7	
			6	11	20:Δ11,14	2	
21	22.08	0.5					
22	23.15	1.2	12	7	22:Δ7,10	16	18:Δ6 + 2C_2→22:Δ10→22:Δ7,10
			11	8	22:Δ8,11	5	
			10	9	22:Δ9,12	70	16:Δ6 + 3C_2→22:Δ12→22:Δ9,12
			9	10	22:Δ10,13	9	

[a] Carbon numbers of both the hydrogenated and original acids (as methyl esters) were determined on DEGS polyester as described in (1). Carbon numbers of hydrogenated dienoic acids showed that the dienoic acids were normal acids of the chain lengths listed.

[b] The carbon number 18.95 was for 18:Δ5,8 (see text).

[c] The carbon number 19.23 was for 18:Δ9,12 (see text).

[d] Represents all isomers of that chain length.

Isolation of 3α, 7α, 12α-Trihydroxycoprostanic Acid From Baboon Bile

TRIHYDROXYCOPROSTANIC ACID (3α, 7α, 12α) (THCA) has been implicated as an intermediate in the biological formation of bile acids from cholesterol (1,2). This compound can be converted to cholic acid by suitably fortified rat liver preparations (3,4), and it has been demonstrated that cholesterol can be converted to trihydroxycoprostanic acid in the alligator (5) and in man (6). The conversion of 26-^{14}C-cholesterol to labeled THCA in the baboon is the subject of this communication.

A young male baboon (4-5 years) which had been maintained on a nutritionally adequate semi-synthetic diet (7) was given a dose of 26-^{14}C-cholesterol (0.025 μc) in corn oil by stomach tube. The animal was fasted for 18 hr, bled by venipuncture and killed. The serum, liver, aorta and bile were taken for analysis. Aliquots of liver and aorta were homogenized in chloroform-methanol (2:1) and the extracts assayed for free and ester cholesterol. The bile (13 ml) was deproteinized with ethanol, and the supernatant fluid concentrated to a small volume and subjected to hydrolysis with NaOH at 125 C under pressure (6). The hydrolysate was acidified with HCl and the bile acids extracted with ethyl acetate.

The total serum cholesterol level and the α and β serum lipoprotein cholesterol levels (Table I) were of the same order of magnitude as those of other baboons fed the same diet (7). The liver contained appreciable quantities of radioactive free (634 cpm/mg) and ester (1872 cpm/mg) cholesterol, but there was no detectable radioactivity in the aorta.

Thin layer chromatography (TLC) of the biliary bile acids on Silica Gel G, using ethyl acetate-acetic acid (95:5) as the developing solvent, yielded major spots corresponding to cholesterol (R_f = 0.69) THCA (R_f = 0.58) and cholic acid (R_f = 0.21). The spots were visualized with an anisaldehyde-H_2SO_4-HOAc spray reagent (10). Another aliquot of the bile acid extract was subjected to TLC, and the areas corresponding to cholic acid and THCA were scraped from the plate and the extracts assayed for radioactivity and subjected to further purification by TLC. The cholic acid thus obtained was not radioactive, which would be expected since the precursor was 26-^{14}C-cholesterol. The THCA fraction contained 2,600 cpm. This material was shown to be identical with authentic THCA by its R_f value in TLC (Fig. 1) and by its retention time when subjected to gas-liquid-chromatography

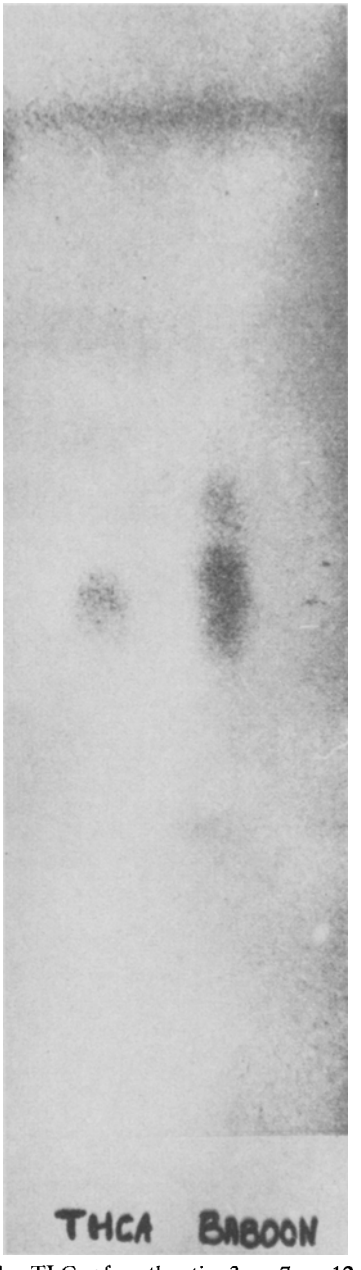

FIG. 1. TLC of authentic 3α-, 7α-, 12α-trihydroxycoprostanic acid (THCA) and of baboon bile. System: Ethyl acetate-acetic acid (95:5) on Silica Gel G. Detection: Anisaldehyde in H_2SO_4-HOAc.

TABLE I
Level and Specific Activity of Serum Cholesterol in a Baboon Fed 26-^{14}C-Cholesterol

Sample	Free[a]		Ester[a]		Total[a]	
	mg/100 ml	cpm/mg	mg/100 ml	cpm/mg	mg/100 ml	cpm/mg
Serum	35	679	79	776	114	703
α Lipoprotein[b]	18	679	41	838	59	740
β Lipoprotein[b]	13	632	38	696	50	608

[a] Method Ref. 8.
[b] Method Ref. 9.

(GLC) (SE-30 supported on 1% QEF).

Pooled bile from 24 baboons, half of whom had been fed either sodium 1-^{14}C-acetate or 4-^{14}C-cholesterol (7), yielded 0.96 g of crude mixed bile acids (676,000 cpm). Preparative TLC on Silica Gel G, using isopropyl ether-isooctane-acetic acid (50:25:30) as the developing solvent, yielded areas corresponding to cholic acid (40,800 cpm) and THCA (15,600 cpm). The two bile acids were shown to be similar to authentic samples by TLC and GLC.

The THCA fraction was further purified by crystallization from ethyl acetate to yield 0.10 mg of pure 3α-, 7α-, 12α-trihydroxycoprostanic acid with a specific activity of 647 cpm/mg. The material had a melting point of 184 C and did not depress the melting point of an authentic sample of THCA.

P. P. SHAH
EZRA STAPLE (deceased)
IRWIN L. SHAPIRO[a]
DAVID KRITCHEVSKY[a]
Department of Biochemistry,
School of Medicine,
University of Pennsylvania,
Philadelphia, Pennsylvania

[a] Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania.

ACKNOWLEDGMENTS

Supported in part by grants AM-07202; HE-03299 and HE-05209 from the National Institutes of Health and Research Career Award 4-K6-HE-734.

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[Received July 12, 1968]

A Rapid Micro Technique for Differentiating Between Iso, Anteiso and Other Mono Methyl Branched Fatty Chains

A VARIETY OF TECHNIQUES are available which give either complete or partial information on the location of a methyl branch in a fatty chain. Techniques discussed in (1) include mass spectra, x-ray diffraction, infrared spectra, oxidative degradation, retention data in GLC, melting points of binary mixtures of branched with normal acids, and others. Nuclear magnetic resonance (2), and x-ray diffraction of single crystals of urea adducts (3) have also been used. Except for GLC, all the above techniques are either complex or require at least milligram amounts of material or expensive apparatus, and the hazards involved in identifying branched fatty chains solely on the basis of GLC retention data have been discussed (4).

This report describes a method capable of differentiating between as little as 1 μg of iso or anteiso fatty chains by degrading them with acidic $KMnO_4$ respectively to acetone or 2-butanone, and analyzing the ketones by GLC on a Poropak QS column. In the iso chain the methyl branch is on the ω minus 1 C-atom (alternately designated as ω2) and in the anteiso chain the methyl branch is on the ω minus 2 C-atom (alternately designated as ω3) where ω is the terminal C-atom of the fatty chain.

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P. P. SHAH
EZRA STAPLE (deceased)
IRWIN L. SHAPIRO[a]
DAVID KRITCHEVSKY[a]
Department of Biochemistry,
School of Medicine,
University of Pennsylvania,
Philadelphia, Pennsylvania

[a] Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania.

ACKNOWLEDGMENTS

Supported in part by grants AM-07202; HE-03299 and HE-05209 from the National Institutes of Health and Research Career Award 4-K6-HE-734.

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The methyl ketones produced define the position of the methyl branch, thus, in the general reaction

$$CH_3(CH_2)_n\text{–}\underset{\displaystyle CH_3}{\underset{|}{CH}}\text{–}(CH_2)_m\text{–}\overset{\displaystyle O}{\overset{\|}{C}}\text{–}OR \xrightarrow[(H_2SO_4)]{KMnO_4} CH_3(CH_2)_n\text{–}\underset{\displaystyle CH_3}{\underset{|}{C}}=O + CO_2 + \text{a variety of acids}$$

where R = H or methyl, and n and/or m = 0,1,2,..., when n = 0, the methyl branch is at ω2, when n = 1, the methyl branch is at ω3, etc. The method described here differs from other successful degradation techniques (1,5,6) in that no organic solvent was used, volatile products formed by oxidation could be quickly removed by distillation, and a GLC stationary phase was used (Poropak QS) capable of separating traces of water soluble organic substances in large amounts of water. Although we do not yet know what the practical limit of the method is, detection of 2-dodecanone from the oxidation of methyl 8-methyl-octadecanoate suggests that it can be used to locate a methyl branch at least as far from the terminal methyl group as ω12.

To develop as rapid a method as feasible, we tested the stability of expected ketones to strong oxidizing conditions. Figure 1 shows

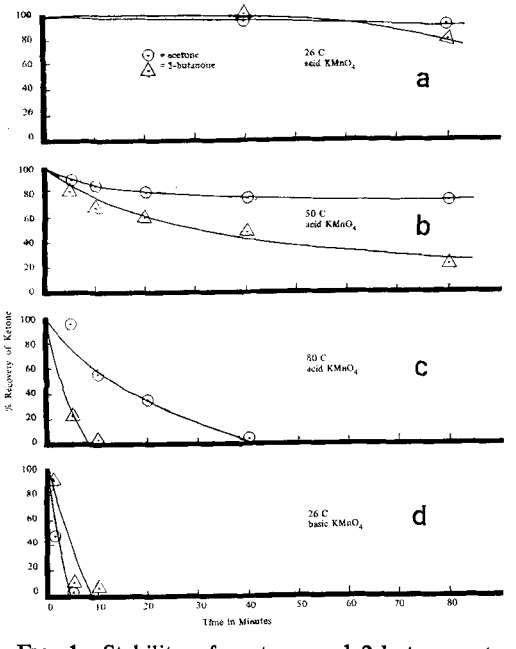

FIG. 1. Stability of acetone and 2-butanone to $KMnO_4$ oxidation. Standard aqueous solutions of ketone (7.9 μg acetone or 6.1 μg 2-butanone) were injected into small tubes containing 80 μl of solution B (Table I, footnote c) for Figure 1a, b and c, or 50 μl of an oxidizing solution consisting of 90 μg $KMnO_4$ and 5.6 μg KOH per μl for Figure 1d. The tubes were sealed and the reaction and analysis carried out as described in Method A of the text.

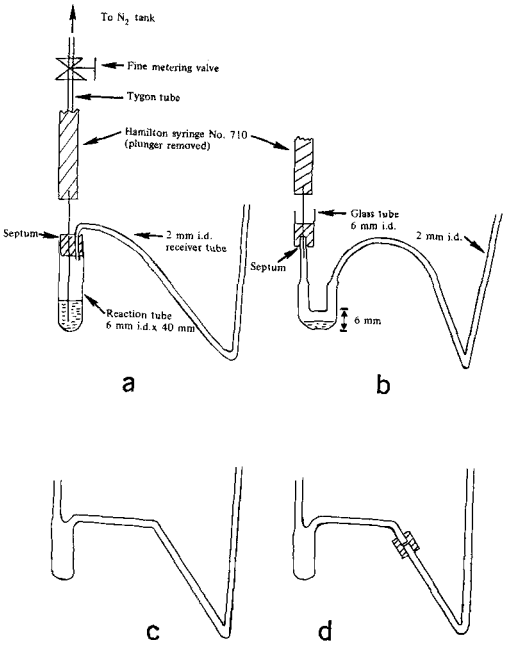

FIG. 2. Apparatus and procedure for Method B. The sample in hexane is introduced into the reaction tube, the hexane removed completely with a stream of N_2, the reaction tube sealed with a septum (w-10, Barber Coleman Co., Inc., Rockford, Ill.) in which the receiver tube is in place. The "V" of the receiver tube is then placed in the cooling bath (dry ice in chloroform-methanol 2:1), and the oxidizing solution introduced through the septum with a 100 μl syringe (No. 710, Hamilton Co., Whittier, Calif.). With the N_2 flow off, the syringe needle connected to the N_2 supply is then pierced through the septum and the needle tip placed into the oxidizing solution. A fine metering valve (Nupro 1S, Nuclear Products Co., Cleveland, Ohio) is used to regulate a flow of N_2 low enough that distinct bubbles can be seen, and finally the constant temperature water bath is raised to immerse the reaction tube. After collection of 10 to 20 μl of distillate (5 to 10 min) the N_2 flow is stopped and the receiver tube disconencted. To mix the distillate the open end of the receiver tube is sealed with a septum and the distillate forced up and down arms of the receiver tube with a syringe. An aliquot is then injected into the gas chromatograph. Fig. 2b, c and d are alternate useful designs of the distillation portion of the apparatus, especially where more quantitative collections of ketones are desired.

TABLE I

Ketones Produced From Iso, Anteiso and Other Branched Fatty Chains by Acidic $KMnO_4$ Oxidation

Reactant			Oxidizing conditions[b]				Product	
Chain	Deriv.[a]	μg	Oxidizing solution[c]	μl	Temp C	Time min	Ketone[d]	Yield[e] %
iso-C_{13}	FA	100	A	100	80	10	acetone	46
anteiso-C_{13}	FA	200	A	100	80	10	2-butanone	10
iso-C_{14}	ME	1	B	20	50	10	acetone	15
iso-C_{14}	ME	10	B	20	50	10	acetone	3
anteiso-C_{15}	FA	100	A	100	80	10	2-butanone	20
anteiso-C_{15}	ME	1000	B	112	95	40	2-butanone	8
anteiso-C_{15}	ME	900	B	100	80	20	2-butanone	12
iso-C_{16}	FA	200	A	100	80	10	acetone	7
iso-C_{16}	ME	1000	B	100	80	40	acetone	4
anteiso-C_{17}	ME	1	B	20	50	10	2-butanone	9
anteiso-C_{17}	ME	1	B	20	80	10	2-butanone	4
anteiso-C_{17}	ME	1	B	20	50	10	2-butanone	15
anteiso-C_{17}	ME	3	B	20	50	10	2-butanone	8
anteiso-C_{17}	ME	10	B	20	50	10	2-butanone	4
anteiso-C_{18}	FA	100	A	100	80	10	2-butanone	18
anteiso-C_{25}	FA	100	A	100	80	10	2-butanone	3
15-Me-C_{18}	ME	400	A	100	80	10	2-pentanone	27
8-Me-C_{18}	ME	500	A	100	80	10	2-dodecanone	?

[a] FA indicates free acid, ME indicates methyl ester. All free acids were gifts of A. W. Weitkamp; methyl esters of iso-C_{14},-C_{16}, and anteiso-C_{15} and -C_{17} were purchased from Applied Science, Inc., State College, Penn.; methyl 15-methyl-octadecanoate and methyl 8-methyl-octadecanoate were gifts of James Cason.

[b] Method B (see text).

[c] Solution A contained 100 μg $KMnO_4$ and 56 μg H_2SO_4 per μl. Solution B contained 60 μg $KMnO_4$ and 85 μg H_2SO_4 per μl.

[d] Ketones were identified by GLC in a Loenco, Inc., Model 160 gas chromatograph with H_2 flame detector; stainless steel column ⅛ in. o.d. × 2 ft. packed with Poropak QS (Waters Associates, Inc., Farmingham, Mass.); He flow 59 ml/min; column temp. 150 C for acetone, 2-butanone and 2-pentanone, and 250 C for 2-dodecanone. Standard ketones were acetone, 2-butanone, 2-pentanone, 2-hexanone, 2-heptanone, 2-octanone, and 2-undecanone (Matheson, Coleman and Bell, Rutherford, N. J.) and 2-nonanone (Aldrich Chemical Co., Inc., Milwaukee, Wis.)

[e] Corrected for a 70% loss during distillation.

that for 5 min, acetone and 2-butanone are reasonable stable to acidic $KMnO_4$ (in at least 40 fold excess for complete oxidation) and to temperatures at least as high as 50 C. Alkaline conditions, however, appeared less promising.

Two methods for carrying out the reaction were studied. In Method A the reactants were sealed in a tube (3 mm i.d. × 40 mm length) and shaken throughout the oxidation period in a constant temperature water bath. The seal was then broken and an aliquot of reaction mixture injected directly into the gas chromatograph. In Method B (Fig. 2) as the volatile reaction products formed they were swept away with N_2 along with some water and this distillate caught in the "V" of a receiver tube was then injected into the gas chromatograph. The distillation could be interrupted to change the receiver or to introduce more oxidant or water into the reaction tube. Method A gave more quantitative results, especially when the ketone produced was water soluble, but the background noise on the gas chromatograph built up to such an extent with time that this method was generally not used. In Method B the volatiles could be concentrated in the relatively small volume of distillate and most of this could be injected into the gas chromatograph, which now could be operated at maximum sensitivity since none of the oxidizing solution was injected. However, in checking the recovery of just the distillation of small amounts of ketone in water (no oxidant present) with any of the set-ups of Figure 2, we could collect no better than 60% (average 30%) of starting ketone even with an additional "V" trap. Method B is restricted to ketones that can be volatilized; however, no difficulty was experienced in volatilizing ketones up to 2-undecanone, and oxidation of methyl 8-methyl-octadecanoate yielded a peak at where 2-dodecanone should have emerged (Table I). If necessary, methanol should be used to solubilize higher molecular weight ketones to give a uniform injection.

Table I lists some of the compounds tested, the oxidation conditions used, and the ketones yielded. Since we could detect nanogram quantities of ketone, even though the average yield was 10% of theoretical, quite sufficient ketone

formed to identify it unambiguously. The two strong oxidizing solutions used revealed no outstanding difference in ketone yield. Negligible amounts of ketones but often increasing amounts of acetic acid formed when additional 10 min distillates were collected or when temperatures higher than 50 C were used. This was true whether or not additional oxidizing solution or distilled water was added to the reaction tube.

N. NICOLAIDES
HWEI C. FU
University of Southern California
School of Medicine
Los Angeles, Calif. 90033

ACKNOWLEDGMENT

This work was supported by research grant AM-10010 from the National Institute of Arthritis and Metabolic Diseases, U.S. Public Health Service and by the Birely Foundation, Los Angeles, California.

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[Received August 7, 1968]

Identity and Configuration of Conjugated Fatty Acids in Certain Seed Oils[1]

C. Y. HOPKINS, M. J. CHISHOLM and J. A. OGRODNIK
Division of Pure Chemistry, National Research Council of Canada, Ottawa, Canada

ABSTRACT

Punicic acid was found in the seed oils of *Cayaponia grandifolia, Trichosanthes cordata* and *T. cucumerina.* α-Eleostearic acid was found in *Momordica cochinchinensis, M. cochinchinensis* variety *mixta* and *M. cymbalaria.* The identity of the conjugated triene acid of *Aleurites trisperma, Garcia nutans* and *Cyclandrophora laurina* was confirmed as α-eleostearic acid (*cis, trans, trans* configuration). The configuration of kamlolenic acid was proved to be *cis*-9, *trans*-11, *trans*-13. The oils of *Aleurites remyi* and *Licania platypus* did not contain any conjugated acid.

INTRODUCTION

Fatty acids with conjugated unsaturation occur in the glycerides of many seed oils (1,2). The mechanism of their biosynthesis is not yet known but a general picture of their occurrence is emerging as more species are studied. An unusual feature is the production of the stereoisomeric acids, α-eleostearic and punicic, by different species of the Cucurbitaceae family (3).

In the present work, six species of Cucurbitaceae and six species of other families were examined. The conjugated acids, where found, were identified with particular attention to the configuration at the double bonds.

EXPERIMENTAL PROCEDURE AND RESULTS

Oils were extracted from the ground seeds at 25 C for examination and by Soxhlet extraction to determine the total oil content (Table I). The solvent, petroleum ether, bp 30-60 C, was removed in a current of nitrogen at 25-35 C. UV spectra were determined in cyclohexane solution and IR spectra as liquid films (neat) or in carbon disulfide solution. Other procedures were as described previously (4,5).

The oils that contained α-eleostearic acid had λ_{max} 262, 272, 283 mμ and ν_{max} (neat) 962, 990 cm^{-1}. Those that contained punicic acid had λ_{max} 265, 275, 287 mμ and ν_{max} (neat) 754, 932, 982 cm^{-1}. The content of conjugated triene acid in each oil was calculated from the UV absorption of the oil and the coefficient E (1% 1 cm) of the pure acids: α-eleostearic, 1766 at 272 mμ; punicic, 1694 at 275 mμ, parinaric 2620 at 307 mμ.

The oils were hydrolyzed and the major conjugated acid was isolated or concentrated by low-temperature crystallization by the usual method (4,5). α-Eleostearic acid was identified as the acid or as its adduct with maleic anhydride by mixture melting point with an authentic sample. The acid melted at 46-47 C and the adduct at 63-64 C. Punicic acid was identified as the acid, mp 43-44 C, and also by stereomutation (5) to β-eleostearic acid, mp 69.5-70.5 C. The identity was confirmed by mixture mp in each case.

α-Eleostearic acid was treated with 2-bromo-4′-phenylacetophenone, giving 4-phenylphenacyl *cis*-9, *trans*-11, *trans*-13-octadecatrienoate as shiny leaflets, mp 67.0-67.5 C.

Analysis. Calculated for $C_{32}H_{40}O_3$: C, 81.31; H, 8.53. Found: C, 81.41; H, 8.72.

Momordica Species

All three *Momordica species* contained α-eleostearic acid (Table I). *M. cochinchinensis* is a common cultivated plant in India. Its seeds were grey-brown, 10-12 mm long, lightly sculptured and with irregular edges. The oil was solid at 20 C. *M. cochinchinensis* variety *mixta* (sometimes referred to as *M. mixta* Roxb.) grows wild in India (6). Its seeds were flat, circular, sculptured, and 20-25 mm in diameter. *M. cymbalaria* (syn. *M. tuberosa*) was examined by Azeemoddin and Rao (7), who determined the properties of the oil and noted that the color tests and drying properties indicated the presence of a conjugated trienoic acid. The seeds were ovoid, 6-7 mm long, and partly covered with a thin, brown seed-coat.

Trichosanthes and Cayaponia

Both species of *Trichosanthes* contained punicic acid (Table I). Seeds of *T. cordata* were black, about 10 mm long, somewhat flattened and pointed at one end. Seeds of *T. cucumer-*

[1] Issued as N.R.C. No. 10589.

TABLE I
Seed Oils of Cucurbitaceae

Species	Oil content, % (air-dry basis)	n_D^{25}	Conjugated acid		Origin of seed
			Identity	Amount in oil, %	
Momordica cochinchinensis Spreng.	47.2[a]	1.5012	α-Eleostearic	59.5	India
M. cochinchinensis Spreng. var. mixta	48.2[a]	1.5022	α-Eleostearic	64.9	India
M. cymbalaria Hook. f.	15.5[b]	1.4957	α-Eleostearic	49.2	India
Trichosanthes cordata Roxb.	23.3[b]	1.4904	Punicic	32.3	India
T. cucumerina L.	25.2[b]	1.5049	Punicic	55.9	India
Cayaponia grandifolia (T. & G.) Small	32.4[b]	1.4942	Punicic	38.7	Arkansas U.S.A.

[a] Kernel (seed less pericarp).
[b] Whole seed incl. pericarp.

ina were grey to brown, about 12 mm long, with irregular edges. This species is very closely related to *T. anguina* L. (8). The oil of *T. cucumerina* was studied by Patel et al. (9), who gave the constants and an analysis of the fatty acids. They reported that the oil contained a conjugated trienoic acid but did not make a definite identification.

Cayaponia grandifolia is a wild plant, found in Arkansas, U.S.A. The seeds were grey, roughly boat-shaped and 7-8 mm long. Three seeds were enclosed in an ovoid capsule which was orange in color and 10-12 mm long.

Emphorbiaceae

The oil of *Aleurites trisperma* was studied in 1935 and 1939 (1). α-Eleostearic acid was reported as the major component by Frahm and Koolhaas (10), who isolated and identified the acid. Its identity was confirmed in the present work by its spectroscopic properties and by preparation of the maleic anhydride adduct. The nuts were obtained from a specimen tree in Florida, U.S.A. The kernels (56% of the nuts by weight) yielded 63.5% of oil, which had n_D^{25C} 1.4953, iodine value 119.2 (Wijs) and 158.2 (by hydrogenation). The oil had E(1% 1 cm) 689 at 272 mμ, equivalent to 39% of α-eleostearic acid, and ν_{max} (neat) 962, 990 cm^{-1}. α-Eleostearic acid was isolated (mp 47-48 C) and converted to its maleic anhydride adduct, mp 62-63 C.

A. remyi Sherff is known only on the island of Hawaii, U.S.A. and may be a natural mutant of *A. moluccana* (11). The nature of the oil was consistent with this opinion. The nuts were roughly spherical, about 30 mm in diameter. The kernels yielded 60% of oil, which had no appreciable UV absorption in the range 230-320 mμ. Thus it had no acids with conjugated unsaturation and was similar to *A. moluccana* oil in this respect (1).

The oil of *Garcia nutans* Rohr. was examined in 1943 by Jamieson and Rose (12), who reported that it contained about 90% of α-eleostearic acid. Confirmation of this result was desirable because the melting point given for the acid (50-51 C) was higher than the mp usually given (47-48 C) and the UV absorption data were not recorded. The present work confirmed the identity of the acid.

Nuts were obtained from Florida, U.S.A. The oil had E(1% 1 cm) 1280 at 272 mμ, equivalent to 72.4% of α-eleostearic acid, and ν_{max} (in CS_2) 957 (m), 985 (vs) cm^{-1}. It yielded α-eleostearic acid, mp 46-48 C, which gave a maleic anhydride adduct, mp 63-64 C, and, on isomerization, β-eleostearic acid, mp 69-70 C. The spectra showed no evidence of conjugated diene or hydroxy acid. The content of α-eleostearic acid was considerably lower than the 90% reported previously (12). This may have been due to some deterioration of the nuts during shipment. Tests of *G. nutans* oil by Westgate (13) indicated that it contained more conjugated acid than *A. fordii*.

Rosaceae

Seeds of *Cyclandrophora laurina* (A. Gray) Kosterm. were obtained from Fiji. This name, confirmed by Jeffrey (14), is synonymous with *Parinari laurina* A. Gray. The seeds (nuts) were of varying size, about 5-8 cm long, ovoid, light brown, with a smooth shell. The oil had λ_{max} 273,283,292,307,322 mμ and ν_{max} (in CS_2) 945(m), 987 (vs) cm^{-1}. The total acids were crystallized from petroleum ether and then twice from ethyl ether. Parinaric acid, mp 84-86 C was obtained. It had E(1% 1 cm) 2620 at 307 mμ. The acids soluble in petroleum

ether yielded crude α-eleostearic acid, mp 41-43 C. It gave the maleic anhydride adduct, mp 62-64 C. There was no evidence of licanic acid. This is very likely the same species studied by previous workers (15,16) under the name *P. laurinum*. The results of the present work are similar to those of Gunstone and Subbarao (16) and confirm the presence of parinaric acid and the configuration of α-eleostearic acid in *Cyclandrophora laurina*.

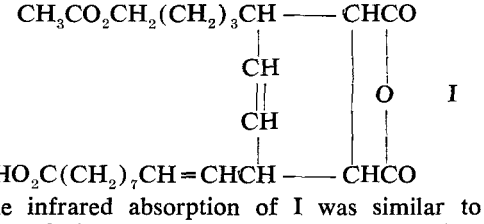
The large nuts of *Licania platypus* (Hemsl.) Fritsch (origin Honduras) had an average weight of 130 g. The hard horn-like kernel was cut into fragments and extracted. It yielded 3.1% of oil, n_D^{25C} 1.4647. The oil had no UV absorption from 230 to 320 mμ except slight end absorption near 230 mμ. Thus this species has no conjugated acids in its seed oil, unlike other *Licania* species.

Configuration of Kamlolenic Acid

Kamlolenic acid, 18-hydroxy-9,11,13-octadecatrienoic acid, has been assumed to have the configuration *cis*-9, *trans*-11, *trans*-13 (1) because the properties due to the triene unsaturation are similar to those of α-eleostearic acid. This does not exclude the alternative form *trans*-9, *trans*-11, *cis*-13, which would have a similar IR spectrum (17).

To establish definitely the configuration, seeds of *Mallotus philippinensis* Muell. Arg., (origin India), were extracted and kamlolenic acid was isolated from the oil. The acid was dissolved in dry ether and acetylated by slow addition of acetyl chloride (18). The acetyl derivative had mp 41-43 C [lit. 43-44 C (18)]. This compound (2 g) was heated in benzene with maleic anhydride (1.8 g) under reflux for 3 hr. The product (crystallized from ether-petroleum ether (1:4), was the adduct 3-9′-carboxy-non-*cis*-1′-enyl-6-4″-acetoxybutyl-cyclohexene-4,5-dicarboxylic anhydride (I), mp 70-71 C.

Analysis. Calculated for C_{24} H_{34} O_7: C, 66.33; H, 7.88. Found: C, 66.41; H, 7.66. A mixture of kamlolenic acid with I melted at 62-64 C.



The infrared absorption of I was similar to that of the corresponding adduct of α-eleostearic acid: ν_{max} (Nujol mull) 945, 1035, 1780 cm^{-1}. There was no appreciable *trans* absorption (ca. 965 cm^{-1}) and no UV absorption peaks in the range 230-320 mμ (no conjugated unsaturation). Thus it was free from unchanged kamlolenic acid and acetate.

Oxidative splitting of the adduct I by von Rudloff's method (19) gave azelaic acid as the only dibasic acid (GLC). Since adduct formation takes place at adjacent *trans* bonds, the C-9 double bond in kamlolenic acid must be *cis* and the configuration is therefore *cis*-9, *trans*-11, *trans*-13.

DISCUSSION

In conjunction with previous reports (3,20), the results show that four species of *Momordica* have α-eleostearic as the major acid. *M. balsamina* L. is exceptional since it contains punicic acid (about 58% of the total acids) (3).

Five species of *Trichosanthes* have been studied. At least three have punicic acid as the major acid, viz., *T. cordata, T. cucumerina, T. anguina* and probably also *T. cucumeroides* (21). *T. dioica* was reported to have "trichosanic" (probably punicic) acid (22) although it was not characterized. A species referred to as *T. kadam* is now classed as *Hodgsonia*. Thus, nearly all *Momordica* species so far have α-eleostearic acid (*cis, trans, trans*) and all or nearly all *Trichosanthes* have punicic acid (*cis, trans, cis*). Punicic acid has been found, so far, only in the plant families Cucurbitaceae and Punicaceae.

The conjugated trienoic acids known to occur in Euphorbiaceae and Rosaceae have the *cis, trans, trans* configuration (α-eleostearic, kamlolenic and licanic). The unusual *trans, trans, cis* form (catalpic acid) has not appeared in these two families although it has been recognized in Bignoniaceae (5).

ACKNOWLEDGMENTS

Some of the determinations were carried out by Miss F. Johnson and R. Lauzon. Advice on some botanical names was given by C. Jeffrey. Seeds were supplied by Paul R. Weissich, Honolulu Botanic Gardens; S. D. Thirumala Rao, Oil Technological Research Institute, Anantapur, India; Julia F. Morton, Coral Gables, Florida, U.S.A.; D. W. Moore, El Dorado, Arkansas, U.S.A.; G. Watkins, Forest Dept. Fiji, and established commercial suppliers.

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[Received June 6, 1968]

Metabolism of Glyceryl 1-^{14}C-Trilinoleate in Rat Testis

MASAMI NAKAMURA and **O. S. PRIVETT**, University of Minnesota,
The Hormel Institute, 801 16th Avenue NE, Austin, Minnesota 55912

ABSTRACT

Animals of the Sprague-Dawley strain were injected intratesticularly with radioactive glyceryl 1-^{14}C-trilinoleate in a sequential experiment and killed at ¼, ½, 1, 3, 6, 12, 24, 36 and 48 hr. Distribution and concentration (specific activity) of radioactivity among the lipid classes and fatty acids were determined. The results showed that radioactive 1-^{14}C-linoleic acid was released from the glyceryl trilinoleate and incorporated throughout the lipid classes. The pattern of the distribution of the radioactivity and specific activities showed that the transformation of linoleic acid between the triglyceride, diglyceride and fatty acid pools was an equilibrium process. Linoleic acid released from glyceryl 1-^{14}C-trilinoleate was converted to higher polyunsaturated fatty acids which were incorporated throughout the lipid classes, and was catabolized as evidenced by the finding of radioactivity in palmitic acid. The main metabolic pools in the interconversion of linoleic acid were arachidonic and 22:5 acids. Small amounts of 20:3 and 22:4 were also detected and had high specific activities indicative of their roles as precursors.

INTRODUCTION

Although common pathways for the net synthesis of glycerides and glycerophosphatides have been fairly completely elucidated (1,14,31), and it has been demonstrated that the acyl chains and the skeletal moieties of these molecules may turn over independently of each other (1,5,11-13,20,21,23,24), pathways and the mode of transformations of fatty acids among the lipid classes have not been delineated. The group of enzymes known generally as the acyl transferases, first demonstrated by Lands et al. (17-19), appear to play an important role in these processes (8-10,20,26,28). Certainly, specificities of these enzymes for the incorporation of fatty acids into 1 and 2 monoacylglycerophosphorylcholine has been well demonstrated (16,20,25). Studies by the authors (22) on the incorporation of radioactive linoleic acid into the lipid classes of rat testes indicated that the fatty acid pool may play a central role in an equilibrium process, whereby incorporation of fatty acids into these compounds may be controlled. In order to test this hypothesis further and to obtain more information on the equilibria in the transformations of fatty acids among the lipid classes a similar study has been made on the metabolism of glyceryl 1-^{14}C-trilinoleate.

EXPERIMENTAL PROCEDURES

Materials and Methods

1-^{14}C-Trilinolein (Spec. Act. 157.5 mc/mM) was obtained from Dhom Products Ltd., Hollywood, California and purified by argentation TLC. The final preparation was chromatographically homogeneous triglyceride, chemically and radiochemically pure as determined by radio GLC of the constituent fatty acids (after interesterification with methanol). This preparation was emulsified with a mixture of equal parts saline and rat serum to give aliquots of 50 μliter with an activity of 1.25×10^6 counts/ min. for injections into the animals.

Radioactivity was measured by scintillation counting with a Packard Tri-Carb Model 3002 dual channel scintillation spectrometer with solutions of PPO-POPOP in toluene or dioxane-water (27). The latter solution was used for analysis of material recovered from chromatoplates in radio-TLC. Counting efficiency for ^{14}C was 81.8% in the toluene scintillation solution and 67.2% in the dioxane-water scintillation solution. Activity measurement obtained in the two solutions are not directly compared. All results of radioactivity measurement are expressed in counts per minute (CPM) corrected to those obtained with the toluene solution.

Determination of the percentage distribution of radioactivity among the lipid classes and fatty acids was carried out as described in previous work (22). The method employed for the determination of specific activities and percentage composition of lipid classes and fatty acids has also been described in detail in this paper (22). The following abbreviations for the lipid classes are used in the present work: cholesteryl esters (CE), glyceryl ether diester (GEDE), triglycerides (TG), free fatty acids

TABLE I
Per Cent Distribution of Radioactivity in Lipid Classes

Time (hr)		¼	½	1	3	6	12	24	36	48
Animals		2	2	2	4	2	4	3	4	3
Wt. of testis (g)		1.72[a] ±0.03	1.58 ±0.00	1.70 ±0.01	1.59 ±0.06	1.59 ±0.03	1.47 ±0.04	1.57 ±0.02	1.56 ±0.02	1.58 ±0.06
Lipid (wt. %)		3.2 ±0.1	3.5 ±0.0	2.9 ±0.0	3.0 ±0.0	2.8 ±0.2	3.1 ±0.02	2.9 ±0.01	3.0 ±0.02	3.1 ±0.0
Recovery of radioactivity (%)		94.9 ±1.2	87.4 ±1.7	80.7 ±3.4	52.1 ±4.2	40.2 ±4.6	27.2 ±1.4	19.0 ±2.8	10.8 ±1.1	6.9 ±1.1
	Wt. %									
Neutral lipid		95.3 ±0.1	88.6 ±1.7	82.3 ±1.1	57.2 ±0.9	43.5 ±1.1	42.3 ±1.1	54.9 ±2.2	43.4 ±2.3	41.5 ±2.9
CE	0.9	0.3 ±0.0	0.2 ±0.1	0.2 ±0.0	0.3 ±0.0	0.9 ±0.6	0.8 ±0.02	1.0 ±0.1	0.9 ±0.1	1.1 ±0.3
GEDE	0.3	0.1 ±0.0	0.1 ±0.0	0.2 ±0.0	0.1 ±0.0	0.5 ±0.0	0.3 ±0.0	0.4 ±0.0	0.5 ±0.1	0.8 ±0.2
TG	7.8	69.8 ±0.3	57.0 ±1.5	52.8 ±1.4	33.1 ±0.8	24.5 ±2.4	21.5 ±1.1	29.3 ±4.8	20.8 ±3.5	24.8 ±3.1
FA	2.9	20.9 ±1.5	27.3 ±0.4	24.7 ±0.8	17.7 ±0.9	11.1 ±0.1	12.5 ±0.9	16.8 ±2.3	15.6 ±2.1	8.1 ±0.4
DG	1.6	2.4 ±0.1	3.5 ±0.2	3.9 ±0.6	5.3 ±0.2	5.9 ±0.6	5.8 ±0.4	5.3 ±0.4	3.8 ±1 4	3.9 ±0.5
Polar lipid		4.7 ±0.1	11.4 ±0.1	17.7 ±1.1	42.9 ±0.9	56.6 ±2.2	57.7 ±1.2	45.1 ±2.2	56.6 ±2.3	58.5 ±2.9
DPG	4.4	0.3 ±0.0	0.7 ±0.1	1.1 ±0.3	1.3 ±0.1	1.7 ±0.1	2.4 ±0.7	1.2 ±0.0	1.1 ±0.2	1.8 ±0.2
PE	27.2	0.6 ±0.1	1.9 ±0.3	3.1 ±0.3	8.2 ±0.1	11.8 ±0.4	14.2 ±0.2	12.2 ±0.8	16.2 ±0.7	15.9 ±0.8
PI + PS	8.1	0.4 ±0.1	0.7 ±0.1	1.1 ±0.1	2.1 ±0.2	3.8 ±0.2	6.0 ±0.2	5.6 ±0.3	7.8 ±0.3	8.5 ±0.6
PC	29.3	2.1 ±0.2	7.3 ±1.4	11.3 ±0.1	30.1 ±0.9	37.3 ±1.9	33.1 ±0.4	24.5 ±1.4	29.5 ±1.5	29.6 ±1.4
SPH.	5.6	0.1 ±0.0	0.2 ±0.0	0.6 ±0.1	0.7 ±0.1	0.5 ±0.1	0.6 ±0.1	0.7 ±0.0	1.0 ±0.1	1.2 ±0.1
LPC	tr.	0.1 ±0.0	0.2 ±0.0	0.3 ±0.1	0.3 ±0.1	0.4 ±0.4	0.5 ±0.1	0.4 ±0.0	0.6 ±0.1	0.7 ±0.04

[a]M ± SE, with two animals, mean ± the deviation between the two values.
[b]Major radioactive components.

(FFA), cholesterol (CH), diglycerides (DG), total neutral lipids (NL), total polar lipids (PL), diphosphatidyl glycerol (DPG), phosphatidyl ethanolamine (PE), phosphatidyl inositol (PI), phosphatidyl serine (PS), phosphatidyl choline (PC), sphingomyelin (SPH) and lysophosphatidyl choline (LPC).

Animals

Adult male rats of the Sprague-Dawley strain (200–225 g) were obtained from the Hormone Assay Laboratory, Chicago, Illinois. The animals were housed in individual metal cages and fed ad lib. a semisynthetic diet (13,22) containing 10% added safflower seed oil. At the end of three weeks the animals were injected intratesticularly with 50 μliter of the emulsion of glyceryl 1-^{14}C-trilinolein. Groups of 2 to 4 animals were killed by withdrawal of blood from their aortas after intervals of ¼, ½, 1, 3, 6, 12, 24, 36 and 48 hr. The testes were excised and frozen on dry ice. Then they were allowed to thaw on the surface, were decapsulated and weighed, and the lipid was extracted as previously described with chloroform-methanol (22). Lipid class composition, percentage distribution of radioactivity and specific activities were determined for individual animals. The lipid extracts of the animals in each group were pooled for fatty acid analysis.

RESULTS

The percentage distribution of radioactivity among the lipid classes and its dissipation over the course of the experiment are shown in Table I. These data showed that radioactive linoleic acid was released from the triglyceride fraction and incorporated into the lipid classes. The percentage distribution of radioactivity in the triglycerides remained more or less constant after about 6 hr, indicating that a steady state

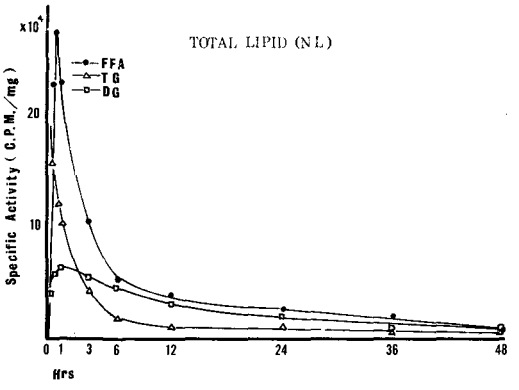

FIG. 1. Time course study of the changes in specific activity of testicular lipids. FFA, free fatty acids; TG, triglycerides; DG, diglycerides.

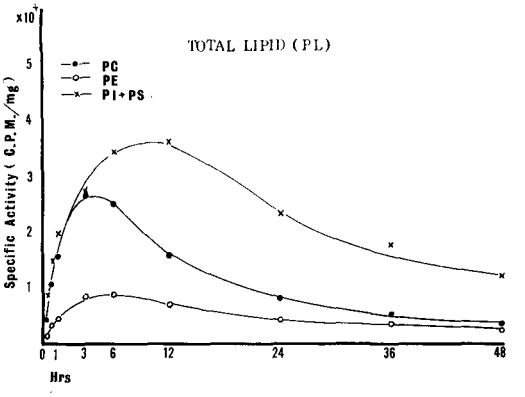

FIG. 2. Time course study of the changes in specific activity of testicular lipids. PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PI, phosphatidyl inositol; PS, phosphatidyl serine.

had been reached. The increase in the percentage distribution of the radioactivity followed a somewhat different pattern in the other lipid class. In some lipid classes, namely PI + PS, SPH and LPC, it increased throughout the experiment. In PC and PE it reached a plateau at about 12 hr after which it appeared to remain fairly constant. In DG radioactivity appeared to peak at about 6 to 12 hr. There was a steady decrease in the percentage distribution of the radioactivity in the free fatty acids after about ½ hr as they were catabolized.

Changes in concentration of the radioactivity (specific activity) of the lipid classes are shown in Figures 1 and 2. The specific activity of the triglycerides decreased quickly as the radioactive linoleic acid was released. The free fatty acids and diglycerides accordingly were the first of the other lipid classes to reach maximal specific activities. PC and PE followed in order with maximal specific activities as they acquired radioactive linoleic acid. The results shown in Table II show that the per cent radioactivity was greatest in the linoleic acid sample. These results showed that 46.9% of the radioactivity was still in linoleic acid after 36 hr when almost 90% of the total radioactivity had been dissipated. However, that conversion to other acids in the linoleic acid family had occurred was demonstrated by the finding of radioactivity in 20:3, 20:4, 22:4 and 22:5 acids. The mass concentration of ω-6-18:3 and ω-6-20:2 were too small to be measured by normal methods of analysis, but the radio-GLC pattern indicated the presence of these acids with very high specific activities.

The radioactivity in palmitic acid apparently occurred by de novo synthesis from radioactive acetate derived from the catabolism of radioactive linoleic acid, or acids to which it was converted.

Figure 3 shows changes in specific activities of the fatty acids of the linoleic acid family in the total lipid. The specific activity of linoleic acid decreased as it was catabolized and converted to other fatty acids. Because linoleic acid was administered as triglyceride, its release occurred over a period of time, as evidenced by the decrease in the percentage distribution of

TABLE II

Per Cent Distribution of Radioactivity in Total Fatty Acids of Testicular Lipids[a]

		Hours							
Acid	Wt. %	¼	½	1	3	6	12	24	36
16:0	32.8	tr.	tr.	0.4	1.0	1.5	2.9	4.4	8.6
18:2	5.0	93.3	86.5	90.2	87.3	78.8	63.6	62.6	46.9
20:3	1.2	0.5	0.7	1.0	1.4	3.2	4.7	4.7	7.3
20:4	14.5	0.3	1.0	0.8	2.6	7.5	10.6	12.6	6.6
22:4	2.5	tr.	tr.	tr.	0.4	0.9	2.4	2.8	5.7
22:5	20.9	tr.	tr.	tr.	tr.	0.3	1.0	0.9	2.3

[a] Pooled samples.

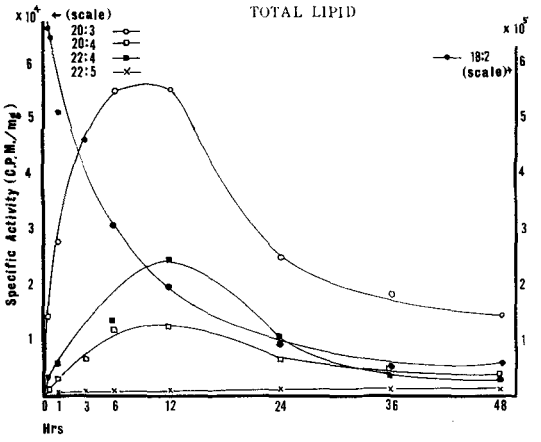

FIG. 3. Time course study of the changes in specific activity of fatty acids in testicular lipids.

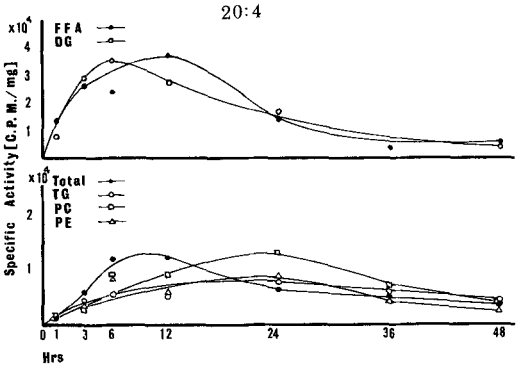

FIG. 5. Time course study of the changes in specific activity of arachidonic acid in lipid classes of testicular lipids. FFA, free fatty acids; DG, diglycerides; TG, triglycerides; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.

radioactivity in the triglycerides during the first 6 to 12 hr (Table I). Thus the normal pattern of product-precursor relationship in the conversion of linoleic acid to other members of this family of acids was masked. However, the pattern of the specific activities indicated precursor roles for 20:3 and 22:4 inasmuch as they had higher specific activities than the acids to which they were converted, 20:4 and 22:5, respectively, and because they were present in only minor amounts. Similar patterns for the specific activities of these acids (20:3, 20:4 and 22:5) were observed in TG, PC and FFA as in the total lipid. In general, the specific activity of the 20:3 was higher and reached a maximal value earlier than the 20:4 to which it was converted. A similar relationship existed between the 22:4 and 22:5, although the specific activity of the 22:5 had not reached a maximum value at the end of the experiment.

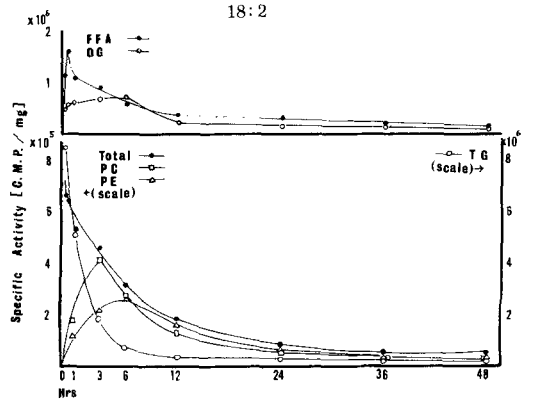

FIG. 4. Time course study of the changes in specific activity of linoleic acid in lipid classes of testicular lipids. FFA, free fatty acids; DG, diglycerides; TG, triglycerides; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.

Figures 4 and 5 show the relationships of the specific activities of the 18:2 and 20:4, respectively, in the lipid classes. The specific activity of linoleic acid decreased in the triglycerides as it was released and metabolized. It also decreased in a similar manner in the other lipid classes after initial increases that occurred at slightly different times. The relationship of the specific activities of arachidonic acid in the different lipid classes (Fig. 5) was typical of the acids produced by interconversion from linoleic acid. The specific activities of these acids appeared to reach maximal values first in the diglycerides and in the free acids, and later, at approximately 24 hr, in the PC, PE and TG.

DISCUSSION

The present study shows that radioactive glyceryl 1-^{14}C-trilinoleate injected into the testes of rats releases the radioactive linoleic acid which is incorporated into all the lipid classes. Since a previous study (22) showed that radioactive linoleic acid injected into rat testes is incorporated into triglycerides, as well as the other lipid classes, it is apparent that transformation of the fatty acids between triglyceride, free fatty acid and diglyceride is an equilibrium process. The leveling out of the percentage distribution of the radioactivity in the triglycerides at about 6 hr (Table I) also indicates

further that the exchange of fatty acids between the TG, DG and FFA pools in an equilibrium process. It is probable that the other lipid classes are likewise in equilibrium with the TG, DG and FFA, particularly PC and PE, because the percentage distribution of radioactivity had a plateau in these compounds in 6 to 12 hr.

The present study does not reveal the nature of the process whereby fatty acids are exchanged between the lipid classes. The low specific activity of LPC seems to preclude it as a precursor to PC but its low specific activity may result if only the β-position in PC is the active site of the exchange reaction for linoleic acid. Determination of the distribution of the fatty acids in PC and studies on the mode of incorporation of radioactive palmitic acid now in progress should yield information on this point. Phosphatidic acid was not separated from DPG but this fraction did not contain sufficient radioactivity to indicate that it could be involved in the pathway leading to the incorporation of triglyceride fatty acids into PC. A similar observation by Reiser et al (23,24) on the processes of lipid adsorption with doubly labeled triglycerides lead to the suggestion that triglycerides may be converted directly to phospholipids. Studies by Collins et al. (5,6) on the metabolism of inorganic phosphate showed that individual molecular species of phospholipids incorporate ^{32}P at rates that depended on the fatty acids they contain. Further studies (29) indicated that different molecular species of phospholipids turn over at different rates. Our studies do not determine whether the fatty acid pool directly, or the diglyceride pool, is the immediate precursor of the acyl chains in PC, but it shows well the intimate relationship existing between these compounds as well as PE in rat testis. Noteworthy in this regard is the work of Bjørnstad et al. (1) showing that the synthesis of PC from the interreaction of diglyceride with cytidine-diphosphoryl-choline is reversible. Possible answers to some of the questions passed by the above observations may be obtained by an extension of the present study with doubly labeled triglycerides or ^{32}P.

In addition to reactions that involve acyl chains are those of the turnover of the base in phospholipids and possibly glycerol in triglycerides that could effect molecular species in a nonspecific manner. Example of such a reaction is the conversion of PE to PC via methylation (1,2,30,31). The mixture of this type of reaction with those involving enzyme specific acylations could account for the apparent lack of more sharply defined molecular species composition than generally observed. Regardless, it is apparent that the fatty acid pool plays an important role in the process and, because TG and DG pools are in active equilibrium with it, they also appear to be important in the overall process.

That some of the minor component phosphatides, notably PI, PS, SPH and LPC continue to increase in percentage distribution of radioactivity slowly over the course of the experiment indicates that either the turnover of fatty acids in these compounds is very slow or that their synthesis occurs mainly by pathways that do not involve directly the free fatty acid-diglyceride-triglyceride pools.

The finding of radioactivity in 20:3, 20:4, 22:4 and 22:5 demonstrates, in accordance with the studies of Davis et al. (7), and Kirschman and Coniglio (15), that linoleic acid undergoes interconversion in the testes of rats. It has generally been assumed that the interconversion of linoleic acid in the testis follows the same pathways and gives the same products as in the liver. Proof of this point was obtained recently in the excellent work of Bridges and Coniglio (3,4). Although 18:3 and 20:2 were not measured in the present study they appeared to be present as indicated by radio-gas chromatography. Likewise, the 20:3 and 22:4 acids detected are probably members of the linoleic acid family and precursors of 20:4 and 22:5, respectively. These acids (20:4 and 22:5) may have specific functions in the testis in that they accumulate in preference to other very closely related members of the linoleic acid family.

ACKNOWLEDGMENTS

This investigation was supported in part by USPHS grants AM 04942 and HE 08214 from the National Institutes of Health.

Brad Westra gave technical assistance.

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[Received August 22, 1968]

Cis-5-Monoenoic Fatty Acids of *Carlina* (Compositae) Seed Oils[1]

G. F. SPENCER, R. KLEIMAN, F. R. EARLE and **I. A. WOLFF**, Northern Regional Research Laboratory,[2] Peoria, Illinois 61604

ABSTRACT

Seed oils of *Carlina corymbosa* L. and *C. acaulis* L. contain *cis*-5-octadecenoic acid as a major fatty acid (21–24%). This acid has not been previously reported as a constituent of Compositae seed oils. The predominant fatty acid in the *Carlina* oils is linoleic (50–52%); lesser amounts ($\leq$ 10% each) of palmitic, stearic and oleic acids are also present. The oil of *C. acaulis* has almost 2% of *cis*-5-hexadecenoic acid; *C. corymbosa* oil includes minor amounts of some oxygenated fatty acids.

INTRODUCTION

In the GLC analysis on a polyester column of methyl esters derived from *Carlina acaulis* L. and *C. corymbosa* L. (Compositae) seed oils, a broad asymmetric peak was observed in the region normally associated with methyl oleate. This paper reports the characterization of the unusual monoenoic ester responsible for this abnormality, as well as the occurrence of an unusual hexadecenoic acid in *C. acaulis* oil and some oxygenated fatty acids in *C. corymbosa* oil.

Carlina is a small genus (20 species) of Eurasian herbs of which the perennial *C. acaulis*, the weather or silver thistle of the Alps, is the only cultivated species (1, 2).

MATERIALS AND METHODS

Samples were prepared for analysis and analyzed for oil content and characteristics as previously described (3). The seed analyzed included pericarp. GLC of the oils and oil fractions was carried out essentially as described for triglycerides by Litchfield et al. (4).

Preparative TLC of *C. corymbosa* oil, on a 1 mm layer of Silica Gel G with a hexane-ether (70:30) solvent system, separated five major areas. These bands were scraped from the plate and the components were eluted from the adsorbent with ether which then was removed under nitrogen on a steam bath. Ultraviolet (UV) analysis of selected fractions was carried out in ethanol on a Beckman DK 2-A spectrophotometer. The five fractions were respotted, along with the whole oil and standards, on an analytical (275 μ layer) plate. This plate was developed as before and then tested for the presence of epoxy groups with picric acid by a procedure similar to that of Fioriti and Sims (5).

Methyl esters were prepared from the unfractionated oils by the method of Metcalfe et al. (6). Esters were analyzed by GLC and TLC (3). Preparative GLC and TLC (7) were used to separate the esters by both chain length and degree of unsaturation. Double bond positions in the unsaturated esters were determined by reductive ozonolysis; GLC was used to identify fragments (8).

Infrared (IR) analyses were carried out in CS_2 in 1 mm NaCl cells on a Perkin-Elmer Model 337 spectrophotometer.

RESULTS AND DISCUSSION

Nonoxygenated Esters

GLC analysis of the methyl esters from *C. acaulis* and *C. corymbosa* seed oils on a polar column (LAC-2-R 446) gave poor resolution between methyl stearate and the 18:1 esters, a condition which suggested the presence of a positional isomer of methyl oleate (9). TLC analysis on silver nitrate-impregnated plates showed no separation of monoenoic esters. The absence of bands in the 10-11 μ region of the IR spectra of the oils and of the ester fractions precluded significant amounts of *trans* unsaturation (10).

After the esters had been separated according to chain length (C_{16} and C_{18}) by preparative GLC, preparative TLC on silver nitrate-impregnated plates was used to separate the C_{18} fractions by degree of unsaturation. Methyl esters from *C. corymbosa* oil contained so little C_{16} monoenoic esters by GLC (Table I) that the C_{16} fraction from this sample was not recovered.

GLC analysis of the reduced ozonides from the C_{18} monoenoic fractions from both samples gave five peaks (Fig. 1) representing C_5 aldehyde ester (AE), C_{13} aldehyde (A), C_9 AE, C_9 A, and unreacted esters. Identifications were based on the retention characteristics of the components on two columns of dissimilar liquid

[1] Presented at the AOCS Meeting, New York, October 1968.

[2] No. Utiliz. Res. Dev. Div., ARS, USDA.

TABLE I
Analytical Data

Source	Seed analysis: Seed wt. (g/1,000)	Protein dry basis N × 6.25 (%)	Oil dry basis (%)	Oil properties: Iodine value (Wijs)	Refractive index 40 (n_D)	HBr equiv.[a]	Per cent composition of esters by GLC: 14:0	16:0	16:1[5]	18:0	18:1[5][b]	18:1[9][b]	18:2[9,12]	18:3	20:0	Oxygenated esters
Carlina acaulis L.	6.6	49	19	119	1.4671	3.1	Trace	8	2[c]	4	24	10	52	Trace	0.1	....
C. corymbosa L.	0.8	37	27	110	1.4663	8.3	0.1	8	Trace	9	21	8	50	0.4	0.5	3

[a] Calculated as per cent epoxyoleic acid.
[b] Calculated from quantities of fragments after reductive ozonolysis. See text.
[c] Includes traces of 16:1[7] and 16:1[9].

phases (8). The relative amounts of the two parent esters (18:1[5] and 18:1[9]), calculated from the response of their ozonolysis fragments (8), are shown in Table I.

The C_{16} fraction from *C. acaulis*, analyzed by the same procedure, gave predominately C_5 AE and C_{11} A from the reduced ozonides. Traces of fragments from two other hexadecenoates (Δ7 and Δ9) were also observed. IR analysis of this fraction in CS_2 also showed no *trans* absorption (10); therefore the 5-hexadecenoate has the *cis* configuration.

The 18:2 fractions from both samples, analyzed by the interrupted ozonization technique for polyenoic esters (8), were methyl linoleate.

Oxygenated Esters

Titration of *C. corymbosa* oil at 55 C for oxirane oxygen (11) indicated higher amounts of HBr-reactive materials in this oil than in *C. acaulis* oil (Table I). Figure 2 shows the analytical TLC of the fractionated *C. corymbosa* oil, as well as the whole oil and standards. *Euphorbia lagascae* oil, which contains mono-, di-, and trivernoloyl triglycerides (12), was used as the epoxy standard. *Dimorphotheca sinuata* and *Coriaria myrtifolia* oils served as the references for glycerides containing acids

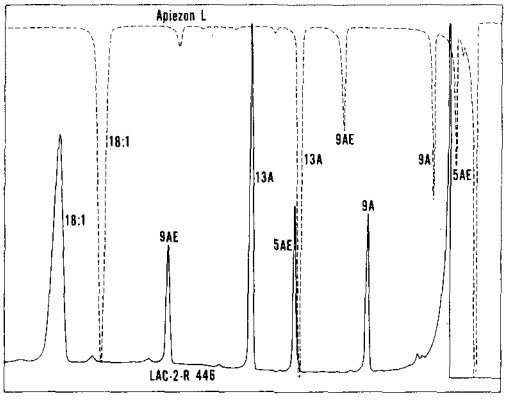

FIG. 1. GLC of reduced ozonides of *cis*-5- and *cis*-9-octadecenoates; A, aldehyde; AE, aldehyde ester. Columns: 5% LAC-2-R 446 on Chromosorb W, 12 ft × ¼ in.; 5% Apiezon L on Chromosorb W, 4 ft × ¼ in. F&M 402 gas chromatograph equipped with flame ionization detectors, temperature programmed 80–200 C at 7.5 C/min. (8).

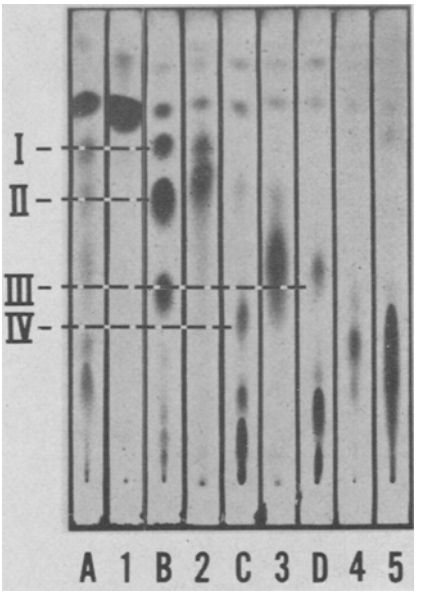

FIG. 2. TLC of *Carlina corymbosa* oil, fractions, and standards on Silica Gel G. Solvent: hexane-ether (70:30). Lanes: A, *C. corymbosa* oil; B, *Euphorbia lagascae* oil; C, *Dimorphotheca sinuata* oil; D, *Coriaria myrtifolia* oil; lane numbers correspond to fraction numbers. Spots: I, monovernoloyl triglycerides; II, divernoloyl triglycerides; IIIB, trivernolin; IIID, monocorioloyl triglycerides; and IV, monodimorphecoloyl triglycerides.

of the hydroxy-conjugated diene type (13,14). The hydroxy-dienoic acids did not give a positive picric acid test.

Peaks from fractions 1 (usual triglycerides), 2 and 3 (Fig. 2) eluted from the GLC column in the C_{50} to C_{56} region. Fraction 2 showed two spots in the monovernoloyl triglyceride region of the TLC plate, both of which gave a positive reaction with picric acid. Fraction 3 had UV absorption at 233 mμ and IR absorption at 10.2 and 10.5 μ. The components comprising this fraction migrated in the monocorioloyl and monodimorphecoloyl triglyceride region of the TLC plate. The UV absorption at 233 mμ indicated the presence of 26% hydroxydienol if calculated as methyl coriolate [methyl 13-hydroxy-*cis*-9-*trans*-11-octadecadienoate (14)] or 21% if calculated as methyl dimorphecolate [methyl 9-hydroxy-*trans, trans*-10,12-octadecadienoate (13)]. The proportion of conjugated diene (approximately the 35% theoretical value) indicated by UV, the IR absorption of the conjugated *cis-trans* type (15), and the migration characteristics suggested that fraction 3 was composed of triglycerides in which one acid was a hydroxy-*cis-trans*-conjugated diene. The presence of *trans-trans* conjugation was, however, not completely excluded. The unusual oxygenated fatty acids in fractions 2 and 3 accounted for the higher HBr titration for *C. corymbosa* oil. Their presence may be analogous to the occurrence of oxygenated acids in *Helianthus* seeds oils (16). *C. acaulis* oil contained a lesser, but measurable amount of HBr-reactive material (Table I); however, GLC and TLC gave no evidence of significant amounts of oxygenated fatty acids.

Fraction 4 showed neither conjugation by UV analysis nor epoxy groups by the picric acid test. The location of the spots (Fig. 2) and the presence of diglycerides in this fraction by direct GLC suggested that the spots represented 1,2 and 1,3 diglycerides. Fraction 5 migrated in the free fatty acid region of the plate and had an IR spectrum similar to that of oleic acid.

Although monoenoic acids with 5,6 unsaturation have previously been found in some Ranunculaceae and Limnanthaceae seed oils (17–20) and a number of highly unsaturated acids with isolated $\Delta 5$ unsaturation have been identified in *Ephedra campylopoda* seed oil (7), we believe that the *Carlina* seed oils described here contain the highest percentages of *cis*-5-octadecenoic and *cis*-5-hexadecenoic acids reported in seed oils and that this is the first record of these acids in the Compositae family. Since the unusual octadecenoic acid was found in the oils of two different species from different countries (*C. acaulis* from Yugoslavia and *C. corymbosa* from Spain), it may be characteristic of the genus. Except for the *cis*-5 acids and minor amounts of oxygenated acids in the oil of *C. corymbosa*, the other fatty acids in the two species are those normally found in seed oils.

ACKNOWLEDGMENTS

Q. Jones, USDA Crops Research Division, Beltsville, Md., for seed procurement and identification. Mrs. M. H. Rawls and Mrs. M. A. Spencer for technical assistance.

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[Received September 16, 1968]

Studies on Liver Phosphatidyl Cholines: II. Effect of Sex, Age and Species Differences on Phosphatidyl Cholines From Liver Mitochondria and Microsomes

JAMES E. MILLER and **W. E. CORNATZER**, Guy and Bertha Ireland Research Laboratory, University of North Dakota, School of Medicine, Grand Forks, North Dakota 58201

ABSTRACT

Liver mitochondrial and microsomal phosphatidyl cholines differing in the degree of unsaturation of their fatty acids have been separated into four fractions by silver ion silica gel TLC. The levels of the four phosphatidyl choline fractions were determined for male and female rats and mice, fetal and young rabbits, and female hamsters and guinea pigs. The sum of phosphatidyl choline fractions 1, 2, and 3 of mitochondria and microsomes was greater in the female rat than in the male rat with the difference being a reflection of a higher level of fraction 3 which contains arachidonic acid. The female rat has greater concentration of phosphatidyl choline fractions 1 and 3 of mitochondria. Similar results were seen in mouse liver microsomes but not in mitochondria. The levels of the individual four fractions varied from species to species. No change occurred in the levels of the phosphatidyl choline fractions of fetal (-9 and -3 days) rabbits, but an increase was seen in the level of fraction 4 between day 3 and day 35 in both the mitochondria and microsomal fractions of liver. The concentration of mitochondrial and microsomal protein, total phospholipid and total lecithin phosphorus were determined in rat, mouse, hamster and guinea pig. The total phospholipid phosphorus/protein (μg/mg) of microsomes was greater in all species than that observed in mitochondria. Liver microsomes contain 45–50% of total phospholipid phosphorus as lecithin whereas mitochondria contains 32–37%. The fatty acid patterns of mitochondria and microsomal phosphatidyl cholines were determined and the ratio of palmitate to stearate was greater than two for mice and hamsters and approximately 0.5 for rat and guinea pigs.

INTRODUCTION

Phosphatidyl cholines with polyunsaturated fatty acids are essential to certain enzymes (1,2) and are components of the structural membranes of the subcellular fraction of the cell (3,4). Recent studies have proven that the phosphatidyl cholines represent a metabolically, heterogeneous class of phospholipids (5–7). Biosynthesis of phosphatidyl choline is influenced by the sex of the animal at least in the case of rats (8,9), but this remains to be established for other animals. Arvidson (10) has overcome one of the difficulties encountered in the study of phosphatidyl cholines by introducing a technique for the fractionation of phosphatidyl cholines according to the degree of fatty acid unsaturation. In the same studies he demonstrated a species difference in the composition of whole liver phosphatidyl cholines. Recently evidence for the increase in smooth surface endoplasmic reticulum following birth has been presented (11,12). Miller and Cornatzer (13) have shown a progressive increase in concentration (μg phospholipid phosphorus/mg protein) of microsomal phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl inositol during development (-12, -9, 0, +2, +9 and +14 days of age). However, the concentration of the individual phospholipids of mitochondria does not change during development.

The purpose of this study was to establish the composition of phosphatidyl cholines of liver mitochondria and microsomes with respect to the sex and species of animals and to examine the phosphatidyl cholines of liver mitochondrial and microsomal fractions of fetal and newborn animals (rabbits).

MATERIALS AND METHODS

Reagents

The reagents used in these experiments were Analytical Reagent grade. The organic solvents were either spectranalyzed or nanograde.

Experimental Animals

To investigate the effect of sex differences on liver mitochondria and microsomal phosphatidyl cholines, a study was made using male and female Sprague-Dawley rats (Sprague-Dawley, Inc., Madison, Wis.) and male and female

albino mice. The rats and mice were allowed free access to food (Purina Laboratory Chow, Ralston Purina Company, St. Louis, Mo.) and water.

Age studies were carried out using New Zealand White Rabbits (Gopher State Caviary, St. Paul, Minn.). Rabbits were maintained on Purina Rabbit Chow Checkers and water, ad lib. After the desired gestation interval, the pregnant female rabbit was killed by stunning and bleeding, and the fetal rabbits were removed quickly. Fetal (-9, and -3 days) and young rabbits (3 and 35 days) were killed by decapitation.

When the effect of species differences on phosphatidyl choline levels was investigated, the composition of phosphatidyl choline was determined using liver mitochondria and microsomes from female rats, hamsters (Simonson Laboratories, Inc., White Bear Lake, Minn.) and female mice. Hamsters were fed Purina Laboratory Chow, and guinea pigs received Purina Guinea Pig Chow.

Preparation of Mitochondrial and Microsomal Fractions

All animals were killed by decapitation. The livers were removed, rinsed with cold water, blotted and weighed. The livers were homogenized in 0.25 M sucrose using a Potter-Elvehjem homogenizer with a teflon pestle. The mitochondrial and microsomal fractions were isolated by differential centrifugation (14). The nuclear fraction was obtained by centrifugation at 800 × *g* for 10 min. The nuclear fraction was washed one time, and the mitochondrial fraction was obtained from the pooled supernatant solution by centrifugation at 14,500 × *g* for 10 min. The mitochondrial preparation showed less than 1% microsomal contamination as determined by the analysis for microsomal glucose-6-phosphatase. The mitochondrial fraction was washed two times, and the pooled supernatant solutions were centrifuged at 78,450 × *g* for 45 min to sediment the microsomal fraction. The microsomal preparation was assayed for succinate dehydrogenase and showed a 1–4% mitochondrial contamination. The protein content of mitochondrial and microsomal fractions was determined by a modified Biuret method (15,16).

Extraction of Lipids and Isolation of Phosphatidyl Cholines

The method of Folch et al. (17) was employed to extract and purify lipids from mitochondria and microsomes. Lipids were stored in a dilute chloroform solution under dry nitrogen at –18 C. The total phospholipid phosphorus (18,19) was determined on an aliquot of the chloroform solution. Phosphatidyl cholines were isolated from the lipid extract by TLC (20). Phospholipids were identified by comparison with purified phospholipid standards. Plates were sprayed with a 0.008% rhodamine 6G solution and viewed under ultraviolet light to identify and outline the band of gel containing the phosphatidyl cholines. After the silica gel containing the phosphatidyl choline had been scraped into a flask containing 20 ml of chloroform-methanol (2:1 v/v), the phosphatidyl cholines were eluted from the gel by filtration of the chloroform-methanol solution with the aid of a sintered glass funnel (medium porosity). The gel was washed twice with chloroform-methanol-water (200:97:3) and once with methanol. Quantitative recovery of phosphatidyl choline was possible with this elution procedure. Recovery values represented 94% of the total phosphorus. The filtrate was washed with 0.2 volumes of 0.04% calcium-chloride. A dilute solution of the phosphatidyl choline extract in chloroform was stored under dry nitrogen at –18 C. On an aliquot of the chloroform solution the total phosphatidyl choline phosphorus (18,19) was determined and the per cent of total lecithin phosphorus per total phospholipid phosphorus was calculated. Fractionation of the phosphatidyl choline fractions were carried out TLC on silica gel H impregnated with silver nitrate (10). The phosphatidyl choline fractions were identified by spraying with a 0.01% methanolic solution of 2,7-dichlorofluorescein and viewing under ultraviolet light (10). Each phosphatidyl choline fraction gave a positive test for choline (21).

Phosphorus was determined by the method of Fiske and Subbarow (19) after elution of the phosphatidyl choline from silica gel impregnated with silver nitrate by the following series of solvents: chloroform-methanol (2:1 v/v); chloroform-methanol-water (200:97: v/v/v); methanol-water (97:3 v/v); and methanol. Recovery values represented 98% of the phosphorus applied to silica gel impregnated with silver nitrate. The per cent of the total lecithin phosphorus in each phosphatidyl choline fraction is similar to that reported (22).

Fatty Acid Determinations

Methyl esters of the phosphatidyl choline fatty acids were prepared by the method of Morgan et al (23). Fatty acids were analyzed using a Barber-Colman Model 10 Gas Chromatography with a ^{90}Sr detector as described previously by Glende and Cornatzer (24). Identifi-

TABLE I

Protein and Total Phospholipid Levels of Liver Mitochondria and Microsomes from Male and Female Rats and Mice

	Rat		Mouse	
	Male	Female	Male	Female
Number of animals	6 (6)[a]	6 (6)	24 (4)	16 (4)
Body weight (g)	215	183	31	25
Liver weight (g)	10.4	7.3	1.6	1.4
Mitochondria:				
Milligram of protein per gram of liver	42 ±4[b]	46 ±4	44 ±2	49 ±3
Microgram of total phospholipid per milligram of protein	6.0 ± 0.3	6.4 ± 0.6	7.1 ± 0.4	6.8 ± 0.9
Microsomes				
Milligram of protein per gram of liver	58 ±3	55 ±6	36 ±9	46 ±3
Microgram of total phospholipid per milligram of protein	11.5 ± 0.7	12.2 ± 1.5	14.1 ± 3.2	12.9 ± 0.5

[a]Values in parenthesis indicate the number of determinations.
[b]Numbers preceded by ± are standard deviations.

cation of the methyl ester derivatives of the fatty acids were identified by comparing the retention ratios (relative to methyl palmitate) to those obtained with standards (Applied Science Laboratories Inc., State College, Pa.). The linearity of the detector was verified by quantitating a mixture of fatty acid methyl esters. The fatty acid composition of each phosphatidyl choline fraction is similar to that reported (22). Docosahexaenoic, oleic, linoleic and arachidonic were the major unsaturated fatty acids in these four phosphatidyl choline fractions. Fraction 1 contains 44.6% docosahexaenoic acid, 1.9% oleic acid, 4.8% linoleic and 6.1% arachidonic acid. Fraction 2 contains 14.6% oleic acid, 10.1% linoleic acid and 26.4% arachidonic acid. Fraction 3 contains 5.3% oleic acid, 15.4% linoleic acid and 38.2% arachidonic acid. Fraction 4 contains 19.2% oleic acid, 28.5% linoleic acid and 1.2% arachidonic acid.

RESULTS

Data given in Table I for protein and total phospholipid from mitochondria and microsomes of rat and mouse liver demonstrate that the sex of the animal does not alter these subcellular constituents to any great extent with only a slightly lower level of protein being observed in liver microsomes of the male mouse.

The results for the phosphatidyl cholines from the different sexes are presented in Table

TABLE II

Comparison of Phosphatidyl Choline Fractions From Liver Mitochondria and Microsomes of Female and Male Rats and Mice[a]

Species	Sex	No. of animals	Phosphatidyl choline phosphorus: Per cent of total phospholipid phosphorus	Phosphatidyl choline fractions (Per cent of total): Fraction 1	Fraction 2	Fraction 3	Fraction 4
Mitochondria							
Rat	Male	16 (16)[b]	32 ±4[c]	10.9 ±2.7	8.5 ±1.4	40.2 ±4.6	40.2 ±5.9
	Female	6 (6)	36 ±3	13.7 ± 1.7[d]	7.9 ±0.8	52.7 ± 3.2[d]	25.7 ± 3.6[e]
Mouse	Male	24 (4)	35 ±6	18.0 ± 0.4	7.5 ± 0.9	31.0 ±2.0	43.5 ±2.9
	Female	16 (4)	34 ±5	14.7 ± 0.6[d]	6.3 ± 0.5[e]	34.5 ±4.8	42.4 ±3.8
Microsomes							
Rat	Male	15 (15)	49 ±5	9.9 ± 1.8	8.3 ± 2.9	39.8 ± 3.9	42.1 ± 4.2
	Female	6 (6)	45 ±3	7.5 ± 1.4[d]	6.6 ± 2.0	53.3 ± 5.0[d]	32.5 ± 6.9[d]
Mouse	Male	24 (4)	43 ±4	14.6 ± 2.4	6.7 ± 1.4	24.6 ± 4.7	59.0 ± 7.1
	Female	16 (4)	50 ±4	11.4 ± 1.7[f]	5.3 ± 0.9	35.3 ± 7.9[f]	45.9 ± 5.5[e]

[a]Fraction 1 of liver microsomes contains 44.6% docosahexaenoic acid, 1.9% oleic acid, 4.8% linoleic and 6.1% arachidonic acid. Fraction 2 contains 14.6% oleic acid, 10.1% linoleic acid and 26.4% arachidonic acid. Fraction 3 contains 5.3% oleic acid, 15.4% linoleic acid and 38.2% arachidonic acid. Fraction 4 contains 19.2% oleic acid, 28.5% linoleic acid and 1.2% arachidonic acid.

[b]Values in parenthesis represent the number of determinations.

[c]Numbers preceded by ± are standard deviations.

The test of significance was applied to the difference between mean values for female and male animals. Probability for occurrence of this difference was: [d]$P < 0.005$; [e]$P < 0.025$; [f]$P < 0.05$.

TABLE III

Phosphatidyl Choline Fractions From Liver Mitochondria and Microsomes of Fetal and Young Rabbits

Age (days)	No. of animals	Body (g)	Liver (g)	Phosphatidyl choline phosphorus: Per cent of total phospholipid phosphorus	Phosphatidyl choline fractions (Per cent of total): Fraction 1	Fraction 2	Fraction 3	Fraction 4
Mitochondria								
-9	24 (4)[a]	6.2	0.5	39 ± 2[b]	5.5 ± 1.4	9.5 ± 3.1	23.2 ± 8.9	61.7 ± 9.3
-3	16 (4)	37.1	2.2	41 ± 1	4.7 ± 0.8	10.2 ± 2.5	24.4 ± 2.6	60.5 ± 2.3
+3	11 (4)	76	2.7	32 ± 2	5.1 ± 2.0	7.7 ± 2.6	26.7 ± 2.4	60.4 ± 5.9
+35	5 (5)	785	41.3	36 ± 2	2.7 ± 1.0	5.0 ± 1.1	15.6 ± 2.0	76.5 ± 3.0
Microsomes								
-9	24 (4)			41 ± 4	4.4 ± 0.9	6.0 ± 1.0	20.3 ± 5.2	69.2 ± 3.6
-3	5 (18)			48 ± 4	4.1 ± 0.6	7.5 ± 1.4	24.4 ± 4.0	64.0 ± 3.9
+3	4 (11)			47 ± 3	3.7 ± 0.3	5.0 ±0.9	25.7 ± 6.2	65.6 ± 6.8
+35	5 (5)			52 ± 3	1.8 ± 0.5	2.2 ± 0.5	14.0 ± 2.0	81.7 ± 2.4

[a]Values in parenthesis represent the number of determinations.

[b]Numbers preceded by ± are standard deviations.

II. The liver mitochondria of the female rat has a statistically significant greater concentration of phosphatidyl cholines fractions 1 and 3 than the male. These fractions contain large amounts of polyunsaturated fatty acids. The mitochondria of liver from male rats contains 29.4% of arachidonic acid in fraction 2 and 36.4% in fraction 3. The liver microsome contains 26.4% of arachidonic acid in fraction 2 and 38.2% in fraction 3. Fraction 3 of mitochondria and microsomes is statistically significantly greater in the female rat as compared to the male rat. Similar results were seen in mouse liver microsomes but not in mitochondria. The concentration of fraction 4 of mitochondria and microsomes is significantly decreased in the female of the rat and mouse. The male mouse has higher levels of fraction 1 of mitochondria and microsomes.

The phosphatidyl choline and phosphatidyl choline fractions from the liver mitochondrial and microsomal fractions of fetal and young rabbits in Table III are depicted as per cent of the total phospholipid and of total phosphatidyl choline respectively. It would appear there is no change in microsomal or mitochondrial phosphatidyl choline fractions during fetal development (-9, -3 days) and postnatal development (+3 days). The decrease in the level of fractions 1, 2 and 3 observed in 35-day-old rabbits suggests the degree of unsaturation decreased between day 3 and day 35. At day 35 the young rabbit has nearly 80% of its liver phosphatidyl choline of mitochondrial and microsomal in fraction 4.

Comparative data for protein and total phospholipid levels of liver mitochondrial and microsomal fractions from female rats, mice, guinea pigs and hamsters are given in Table IV. The concentration of total phospholipid phosphorus/protein (μg/mg) of microsomes was greater than that observed in mitochondrial in

TABLE IV

Comparison of Protein and Phospholipid Concentrations From Liver Mitochondria and Microsomes of Different Female Species

	Species: Rat	Mouse	Hamster	Guinea pig
No. of animals	6	4	6	6
Body weight (g)	183	25	118	346
Liver weight (g)	7.3	1.4	5.8	15.3
Mitochondria:				
Milligram of protein per gram of liver	46 ±4[a]	49 ±3	37 ±8	47 ±4
Microgram of total phospholipid per milligram of protein	6.4 ± 0.6	6.8 ± 0.9	5.4 ± 0.5	5.5 ± 0.5
Microsomes				
Milligram of protein per gram of liver	55 ±6	46 ±3	63 ±15	60 ±4
Microgram of total phospholipid per milligram of protein	12.2 ± 1.5	12.9 ± 0.5	9.0 ± 2.2	15.0 ± 1.4

[a]Numbers preceded by ± are standard deviations.

TABLE V

Comparison of Phosphatidyl Choline Fractions From Liver Mitochondria and Microsomes of Different Female Species

Species	No. of animals	Phosphatidyl choline phosphorus: Per cent of total phospholipid phosphorus	Phosphatidyl choline fractions (Per cent of total): Fraction 1	Fraction 2	Fraction 3	Fraction 4
Mitochondria						
Rat	6	36 ± 3	13.7 ± 1.7	7.9 ± 0.8	52.7 ± 3.2	25.7 ± 3.6
Mouse	4	34 ± 5	14.7 ± 0.6	6.3 ± 0.5	34.5 ± 4.8	42.4 ± 3.8
Hamster	6	37 ± 3	10.0 ± 1.9	7.6 ± 1.3	19.2 ± 2.3	62.3 ± 4.2
Guinea Pig	5	32 ± 1	2.6 ± 0.5	2.7 ± 0.8	13.8 ± 1.0	80.9 ± 1.6
Microsomes						
Rat	6	45 ± 3	7.5 ± 1.4	6.6 ± 2.0	53.3 ± 5.0	32.5 ± 6.9
Mouse	4	50 ± 4	11.4 ± 1.7	5.3 ± 0.9	35.3 ± 7.9	45.9 ± 5.5
Hamster	6	55 ± 1.2	9.1 ± 1.6	6.6 ± 1.4	25.6 ± 4.9	58.7 ± 4.6
Guinea Pig	5	50 ± 2	1.8 ± 0.4	2.1 ± 0.7	13.0 ± 2.3	82.9 ± 2.7

all species. The results given in Table V demonstrate the differences that exist for the phosphatidyl choline fractions and show the similarity for phosphatidyl choline with respect to total phospholipid. Liver microsomes contain 45–50% of the total phospholipid phosphorus as lecithin whereas mitochondria contain 32–37%. Each species has a characteristic pattern for the phosphatidyl choline fractions which is quite similar for liver mitochondria and microsomes. If fractions 1, 2 and 3 are considered as a group which contains the polyunsaturated fatty acids, the rat has approximately 70% of the total phosphatidyl choline in this form. While the mouse has 60%, the hamster about 40%, the guinea pig has slightly less than 20%. In Tables VI and VII fatty acid compositions of microsomal and mitochondrial phosphatidyl choline for the different species are given. Only the major fatty acids have been included. The phosphatidyl choline fatty acid data for mitochondria and microsomes from the liver of a given species are very similar. The rat and guinea pig have a palmitate-stearate ratio of 0.4 to 0.5 while for the other species the ratio is approximately 2.0 to 2.3.

TABLE VI

Fatty Acid Composition of Microsomal Phosphatidyl Cholines From the Livers of the Female Rat, Mouse, Hamster and Guinea Pig

Fatty acid	Species: Per cent of total fatty acids by weight: Rat (6)[b]	Mouse (4)	Hamster (6)	Guinea Pig (6)
14:0[a]	0.2 ± 0.0[c]	t	0.1 ± 0.0	0.1 ± 0.0
15:0	0.1 ± 0.0	t	0.1 ± 0.0	0.2
16:0	14.7 ± 0.8	31.6 ± 1.8	27.3 ± 2.1	11.4 ± 0.8
16:1	1.2 ± 0.4	2.8 ± 0.3	3.8 ± 1.2	1.1 ± 0.1
18:0	29.9 ± 2.3	13.9 ± 1.3	13.7 ± 2.2	29.9 ± 0.9
18:1	7.2 ± 1.2	12.6 ± 1.1	14.3 ± 3.3	14.8 ± 1.0
18:2	14.1 ± 1.2	15.6 ± 0.8	21.8 ± 1.2	34.0 ± 0.9
20:3	0.7 ± 0.5	1.5 ± 1.0	1.5 ± 1.3	1.2 ± 0.7
20:4	25.5 ± 4.2	13.9 ± 0.7	8.7 ± 1.4	3.6 ± 0.4
20:6	6.7 ± 1.1	7.9 ± 1.0	7.0 ± 1.9	N.D.[d]

[a]Number of carbon atoms: number of double bonds.

[b]Number of animals.

[c]Numbers preceded by ± are standard deviations.

[d]N.D. signifies not determined; t signifies trace; only major acids are given.

DISCUSSION

Natori (25) demonstrated a sex difference with respect to the incorporation of the methyl group of methionine into liver phosphatidyl cholines. These studies were extended to show that in liver the methylation of phosphatidyl ethanolamine was of quantitative importance (8) and that this pathway was more active in the female rat as compared to the male rat (8,9). Recently estradiol was found to enhance the methylation of phosphatidyl-ethanolamine and increase the formation of lecithins containing long-chain polyene fatty acids (20:4 and higher) (26). The data presented in Table II demonstrates that the liver mitochondria of the female has a greater concentration of phosphatidyl cholines in fractions 1 and 3 than the male rat. Fraction 3 was increased in microsomes of the female liver. It has been demonstrated that these phosphatidyl choline fractions of phosphatidyl cholines are enriched in polyenoic acids (21). This composition data supported the isotope incorporation findings (8,9,25) and the evidence of Lyman et al. (26) that estradiol stimulates the methylation of phosphatidyl ethanolamine with the formation of lecithins enriched in polyenoic acids. Balint et al. (27) have reported that methylation of phosphatidyl ethanolamine provides a lecithin species having polyunsaturated fatty acids.

Phosphatidyl choline in adult mammals is

known to be synthesized by two different pathways. Kennedy and Weiss (28) have demonstrated a pathway by which cytidine diphosphocholine reacts with an α,β-diglyceride to form lecithin. The other system was discovered by Bremer and Greenberg (29), which involves the transfer of methyl groups from adenosylmethionine to intact phospholipids, presumably with phosphatidyl ethanolamine as an acceptor to produce phosphatidyl choline. Rytter et al. (22) have demonstrated that the administration of 1,2-^{14}C-ethanolamine produces an lecithin enriched in polyunsaturated fatty acids with the peak of incorporation of the isotope occurring in fraction 1. 1,2-^{14}C-choline was primarily incorporated into fraction 4. Baldwin and Cornatzer (30) have investigated the enzymatic activity of lecithin biosynthesis by assaying the enzymatic activity of liver choline phosphotransferase and phosphatidylmethyltransferase activity during pre- and postnatal development of the rabbit. There was a progressive increase in enzymatic activity of the liver choline phosphotransferase activity during development. The peak of activity of the choline phosphotransferase was reached in the 50 day old animals. This increase of enzyme activity was three times that observed for the methyl transferring pathway. The data in Table III further support this observation since over 76% of the total lecithin phosphorus was found in fraction 4 of mitochondria and 81% in microsomes. The decrease of the per cent of total lecithin phosphorus of mitochondria and microsomes in fraction 1 that was observed in -9 to +35 day old fetal rabbits substantiates the low enzymatic activity of phosphatidylmethyltransferase, that was seen at this time of fetal development (30).

The data of Table V suggests that the methylation pathway should be more active in the rat than the guinea pig since the rat has approximately 70% of the phosphatidyl cholines within fraction 1, 2 and 3 while the guinea pig has only about 20% in these fractions. Thus the methylation pathway may not be as important in other species of animals as it is in the rat. The per cent of total lipid-P composition of phosphatidyl choline is greater in microsomes than in mitochondria in all species studied (Table V). This observation is in accordance with the findings in normal mammalian tissues (31–33). Further studies with isotopes and enzymes involved in phosphatidyl choline metabolism must be on a comparative level of species to demonstrate the importance of the different pathways of biosynthesis for production of phosphatidyl cholines. It is hoped that these studies will help establish the nature of the phospholipids of lipoprotein found in cellular membranes and aid in the elucidation of species and sex differences observed with respect to lipids in response to fatty liver induction (34–35) or membrane properties such as permeability (36).

TABLE VII

Fatty Acid Composition of Mitochondrial Phosphatidyl Cholines From the Livers of the Female Rat, Mouse, Hamster and Guinea Pig

	Species			
	Per cent of total fatty acids by weight			
Fatty acid	Rat (6)[b]	Mouse (4)	Hamster (6)	Guinea pig (6)
14:0[a]	t[d]	t	0.1 ± 0.1	0.1 ± 0.0
15:0	t	t	0.2 ± 0.1	0.3 ± 0.1
16:0	13.6 ± 1.8[c]	26.8 ± 0.7	23.2 ± 2.1	11.5 ± 1.0
16:1	1.0 ± 0.3	2.2 ± 0.2	3.3 ± 0.9	1.1 ± 0.3
18:0	30.1 ± 2.4	12.0 ± 1.1	11.9 ± 1.3	28.2 ± 1.4
18:1	6.9 ± 0.4	11.0 ± 1.6	14.0 ± 2.4	13.9 ± 1.0
18:2	11.9 ± 1.1	13.9 ± 1.8	20.3 ± 2.4	35.1 ± 1.8
20:3	0.4	2.9 ± 0.9	2.4 ± 1.3	1.3 ± 0.5
20:4	26.1 ± 1.2	16.8 ± 1.5	11.7 ± 1.8	1.3 ± 0.5
22:6	7.5 ± 1.3	12.4 ± 3.9	10.5 ± 3.8	0.7

[a]Number of carbon atoms: number of double bonds.

[b]Number of animals.

[c]Numbers preceded by ± are standard deviations.

[d]t signifies trace quantities; only the major acids are given.

ACKNOWLEDGMENT

This work was supported in part by a grant from the American Cancer Society P-392B and is part of a thesis submitted by J. E. Miller in partial fulfillment of the requirements for the degree of Doctor of Philosophy. J. E. Miller was supported by a National Institutes of Health Training Grant Fellowship 2T1-GM-565-6.

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[Received August 2, 1968]

Liver Retinal[1] Reductase and Oxidase Activities in Rats Exposed to Low Environmental Temperature[2]

P. R. SUNDARESAN[3] and **DONALD G. THERRIAULT**, Biochemistry and Pharmacology Laboratory, U.S. Army Research Institute of Environmental Medicine, Natick, Massachusetts 01760

ABSTRACT

The influence of low environmental temperature on retinal reductase and retinal oxidase activities in rat liver was examined after two and four weeks of cold exposure at 5 C. Liver retinal reductase decreased whereas retinal oxidase increased with time in control rats fed a retinol-free diet, then replenished with retinyl acetate and maintained at 25 C. When rats under identical experimental conditions were exposed to 5 C, the decrease in retinal reductase and increase in retinal oxidase were both found to be greater at 5 C than at 25 C. These results support the hypothesis that increased metabolic rate from exposure of the animal to a cold environment increases the requirement for retinoic acid and decreases the storage of retinol. Even though changes in total liver retinol levels were similar in both groups, an increased utilization of retinol was indicated when the utilization of retinol was expressed as a ratio of total retinol removed from the liver to total weight gain of the animal.

INTRODUCTION

It was previously reported from this laboratory (1) that total liver retinol levels were not affected by exposing rats to a cold environment. However, an increased utilization of retinol was indicated in cold exposed rats, when the utilization was expressed as a ratio of retinol utilization to weight gain of the animal. It is known that exposure of rats to a cold environment results in an increased metabolic rate (2) which is accompanied by a decreased growth rate. Increased metabolic rate hastens the depletion of liver retinol whereas a decreased growth rate retards the depletion of retinol from the liver (3). Since retinal reductase (alcohol: NAD oxidoreductase, E.C. 1.1.1.1) and retinal oxidase (aldehyde: NAD oxidoreductase, E.C. 1.2.1.3) are intimately involved in retinol metabolism, it was considered desirable to study the effect of cold exposure on the activity of these enzymes. The present report deals with results obtained from experiments designed to measure the effect of exposing rats to a cold environment on the activities of these enzymes in liver.

MATERIALS AND METHODS

Male weanling albino rats of the Holtzman strain, weighing between 40–50 g, were fed a purified retinol-free diet (1) ad lib. throughout the experimental period. After four weeks, when the rats ceased to grow (200–240 g), a daily dose (0.1 ml) of an oil solution of retinyl acetate containing 436.9 μg (1270 international units) was administered orally for five days. The rats were then divided into three groups.

Group 1 served as zero-time controls and were immediately killed. Group 2 remained at 25 C, while group 3 was placed in a cold room at 5 C. After two weeks, half the rats from groups 2 and 3 were killed. The remainder were maintained for an additional two weeks and were then killed.

Determination of Retinal, Retinol and Retinyl Ester

All animals were killed by decapitation. The livers were quickly removed and weighed. About 2 g of each liver was cut out and placed in crushed ice for enzyme assay. The remainder of each liver was used for determination of total retinol by ultraviolet absorption using the three-point correction method as described previously (1).

Total retinol from each liver was separated into retinyl esters and retinol by adsorption chromatography on a 10% (v/w) water-deactivated alumina column. Retinyl ester was eluted with 2% acetone-petroleum ether (v/v, bp 40–60 C) while retinol was eluted with 8% acetone-petroleum ether (v/v).

In in vitro experiments retinal was separated

[1] The specific terms retinol, retinyl acetate, retinal and retinoic acid are used in place of vitamin A alcohol, vitamin A acetate, vitamin A aldehyde (retinene) and vitamin A acid, respectively.

[2] A preliminary report was given at the joint meeting of the Biochemical Society, England and the Society of Biological Chemists (India), September 4-9, 1967, Bangalore, India.

[3] Present address: Lipids Laboratory, Research Institute, St. Joseph Hospital, Lancaster, Pennsylvania 17604.

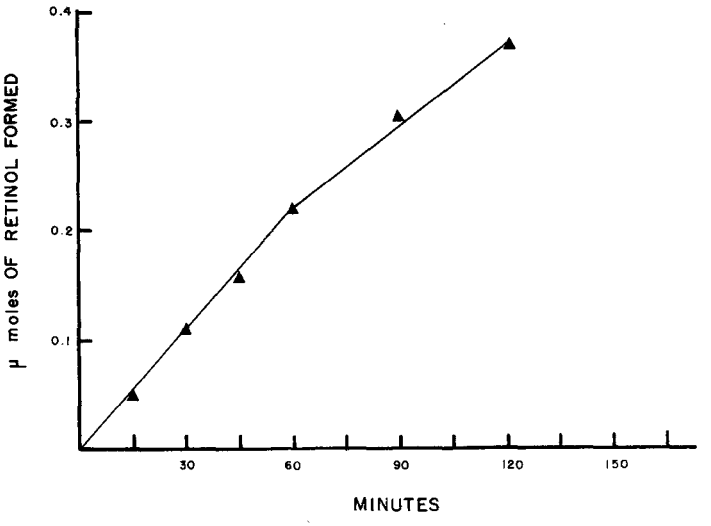


FIG. 1. Time dependence of retinol formation. The incubation mixture in each tube contained in a total volume of 2 ml: 2.5 μmole of retinal in Triton-X-100; 50 μmole of 0.1 M phosphate buffer, pH 7.2; 2.0 μmole of glutathione (reduced); 1.0 μmole of $NADH_2$; 2.0 mg of enzyme protein and 1.0 ml of water. At intervals of 0, 15, 30, 45, 60, 90, and 120 min each tube was removed, the reaction stopped by addition of ethanol and the products separated as described in the text.

from retinol by adsorption chromatography on alumina (4) as described above.

Retinal was measured by the thiobarbituric acid assay of Futterman and Saslaw (5) and retinol was determined in cyclohexane from its extinction coefficient of 1832 at 328 mμ (6). Retinyl ester levels were determined from the difference between total retinol and free retinol in each liver. These values were in agreement with those determined on each retinyl ester fraction by the three-point correction method.

Retinal Reductase and Retinal Oxidase Assay

About 2 g of the liver was homogenized with 6 ml of deionized water in a Kontes glass homogenizer, size c, for 2 min. The homogenate was then centrifuged at 100,000 × *g* for 1 hr. The supernatant fraction was used as the

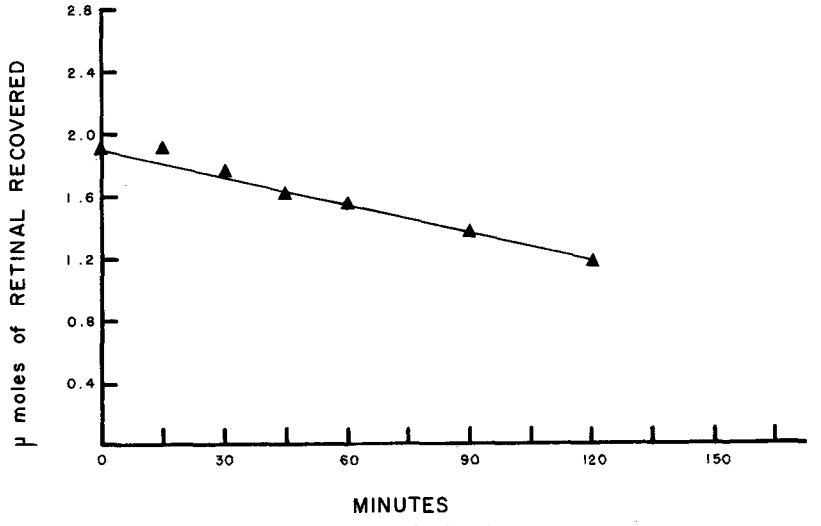


FIG. 2. Disappearance of retinal with time. The incubation mixture is the same as described in Figure 1. At the end of the reaction time, each tube was removed, ethanol was added, and retinal and retinol in the mixture were separated as described in the text.

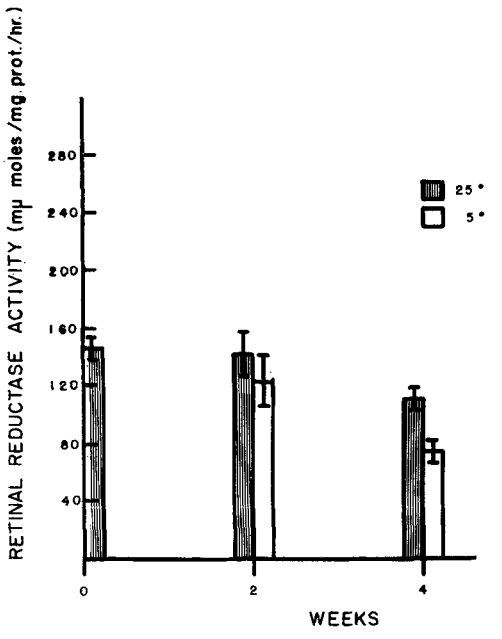


FIG. 3. Retinal reductase activity in liver from rats exposed to 25 C or 5 C. Rats were first depleted of their retinol stores by feeding retinol-free diet and then replenished with retinyl acetate. At intervals of two and four weeks, retinal reductase activity in liver was determined in rats maintained at 25 C or 5 C on retinol-free diet. The mean value of liver retinal reductase activity at 5 C was significantly less ($P < 0.01$) than that from animals at 25 C four weeks after supplementation. Each bar represents the mean value and twice the standard error is represented by the vertical line through the mean.

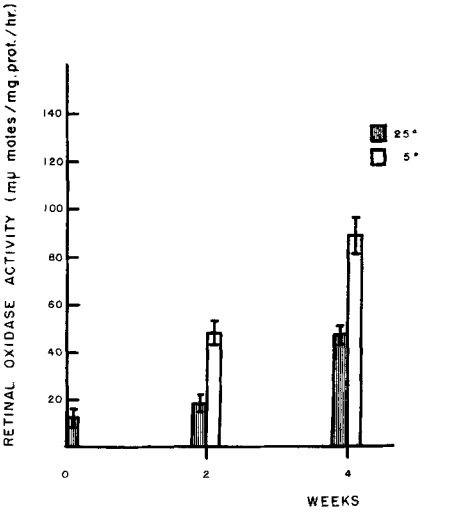


FIG. 4. Retinal oxidase activity in liver from rats exposed to 25 C or 5 C. Rats were prepared as described in Figure 3. At intervals of two and four weeks, retinal oxidase activity was determined in rats maintained at 25 C or 5 C on retinol-free diet. Each bar represents the mean and twice the standard error is represented by the vertical line through the mean. The mean values of retinal oxidase activities of animals at 5 C after two and four weeks were significantly more at $P < 0.01$ and at $P < 0.001$ respectively, compared to those from animals at 25 C.

source of retinal reductase and oxidase activities.

The assay was carried out in a total volume of 2.0 ml containing 0.5 μmole retinal, suspended in 30 mg of Tween 20; 1.0 μmole $NADH_2$; 2.0 μmole glutathione (reduced); 50 μmole 0.1 M phosphate buffer, pH 7.2 (4). The reaction was initiated by adding an aliquot of supernatant containing between 0.6–2.0 mg of protein. The incubation was carried out at 37 C for 1 hr and stopped by adding 5 ml of ethanol.

The reaction mixture was then extracted three times with 25 ml of petroleum ether (bp 40–60 C). The extracts were combined, evaporated to dryness in vacuo and dissolved in a few milliliters of petroleum ether. The reaction products were separated by adsorption on deactivated alumina as described above.

Retinal reductase activity is expressed as the amount of retinol formed/hr/mg protein while retinal oxidase activity is calculated from the difference between the control value and the sum of remaining retinal and retinol values in each experimental tube.

RESULTS

In the present study, the activities of both retinal reductase and retinal oxidase were measured in liver supernatants from animals depleted of retinol stores and subsequently replenished for five days by the administration of retinyl acetate. Retinal reductase activity was measured on the basis of retinol formed from retinal. Figure 1 illustrates the time course of retinol formation. It can be seen that the rate of retinol formation is constant over the first 60 min.

The activity of retinal oxidase was obtained by calculating the difference in the amount of retinal in the control tube to which no enzyme was added and the sum of remaining retinal and retinol formed in each experimental tube. This was necessitated by the inability to detect retinoic acid as a product of the reaction pre-

TABLE I
Ratio of Retinal Reductase to Retinal Oxidase Activity in Rat Liver

Days after supplementation	Temperature 25 C Mean ratio[a]	5 C Mean ratio[a]
0	17.6 ± 1.5[b]	17.6 ± 1.5
14	9.9 ± 0.9	2.8 ± 0.2[c]
28	2.4 ± 0.09	0.9 ± 0.04[c]

[a] Calculated from individual values of retinal reductase and retinal oxidase activities (n = 8).

[b] Standard error of mean.

[c] Values are significantly different from experimental animals at 25 C at $P < 0.01$; Student's t test.

sumably due to its oxidation to other metabolites (7). Recently, retinoic acid has been shown to undergo oxidative decarboxylation both in vivo and in vitro in tissue slices from rat liver and kidney (8–10). On purification of the liver supernatant fraction, it has been possible to identify retinoic acid as the product of the reaction, indicating that retinal oxidase activity is present in the liver supernatant fraction. Deshmukh et al. (11) also measured retinal oxidase activity in liver supernatant fractions by the disappearance of retinal. Figure 2 shows that the disappearance of retinal is linear over a period of 2 hr.

In rats fed a purified retinol-free diet, there is a progressive decrease in liver retinal reductase activity (Fig. 3), whereas retinal oxidase activity showed an increase (Fig. 4). If the changes in enzyme activities in animals exposed to cold are compared with those in control animals, the rate of decrease of retinal reductase and the rate of increase of retinal oxidase are significantly greater in the cold exposed rats. The ratios of retinal reductase activity to retinal oxidase activity were calculated, and are presented in Table I. At zero time, immediately after supplementation with retinyl acetate for five days, the retinal reductase activity far exceeds that of the oxidase. However, the ratio of reductase to oxidase becomes progressively smaller with time on retinol-free diet. If the animals maintained at 25 C are compared with those at 5 C, it is seen that the ratio decreases much more rapidly in the animal exposed to 5 C.

The results of analysis of liver retinyl ester, retinol as well as the animal weight gains at 5 C and 25 C are presented in Table II. There was no significant difference in liver retinyl ester or retinol levels of rats exposed for two and four weeks at 5 C compared with their respective experimental controls at 25 C. However, the weight gains of rats at 5 C were significantly less than those at 25 C. If utilization of total retinol (retinyl ester and retinol) is expressed as a ratio of total retinol removed from liver to weight gain (depletion ratio) as suggested by Nir and Ascarelli (12), then it is observed that there is an increase in total retinol depletion from the liver per gram weight gain in the animals maintained at 5 C (Table II). These results are in agreement with those reported earlier by Sundaresan et al. (1).

DISCUSSION

It has been recently shown that β-carotene is cleaved to 2 molecules of retinal by a soluble enzyme, β-carotene 15, 15′-oxygenase, obtained from the intestine and liver (13,14). Retinal reductase (5,15,16) and retinal oxidase activities (17–20) have both been demonstrated in liver and other tissues. Retinal reductase catalyzes the reversible conversion of retinal to retinol; whereas retinal oxidase causes the irreversible conversion of retinal to retinoic acid.

TABLE II
Effect of Environmental Temperature on Retinyl Ester, Retinol and Weight Gains of Rats Given Retinyl Acetate Orally

Days after supplementation	Temperature	No. of rats	Retinol/liver Ester	Retinol/liver Alcohol	Retinol/liver Alcohol as % of total	Total Weight Gain	Mean Depletion Ratio[e]
			(I.U.)[a]	(I.U.)		(g)	
0	25 C	4	2993 ± 97[b]	148 ± 12	4.7 ± 0.4	.....	.....
14	25 C	6	2602 ± 110[c]	342 ± 40[c]	11.8 ± 1.6	31.0 ± 3.7	9.8 ± 1.6
	5 C	5	2535 ± 72[c]	256 ± 14[c]	9.2 ± 0.6	8.6 ± 5.1[d]	35.0 ± 4.6[d]
28	25 C	6	2347 ± 154[c]	291 ± 18[c]	11.3 ± 1.2	49.3 ± 5.7	17.4 ± 2.3
	5 C	5	2369 ± 86[c]	266 ± 22[c]	10.1 ± 0.9	23.4 ± 6.9[d]	25.6 ± 2.7[d]

[a] 1 international unit (I.U.) is equivalent to 0.344 μg of retinyl acetate or 0.3 μg of retinol.

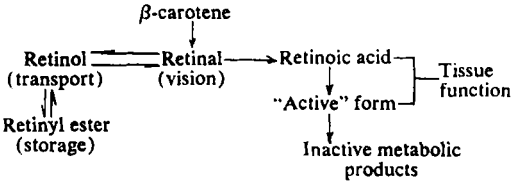
[b] Standard error of mean.

[c] Values are significantly different from zero time controls at 25 C at $P < 0.05$; Student's t test.

[d] Values are significantly different from comparable animals at 25 C at $P < 0.05$; Student's t test.

[e] Mean depletion ratio = total retinol depletion/total weight gain.

The participation of retinal in visual processes has been well elucidated (21). Retinal has also been detected in liver and other tissues (22). Its biological activity in rat is about 90% of that of *all-trans* retinyl acetate (23). Retinoic acid, which is formed from retinal by irreversible oxidation, can replace retinol in biological processes such as growth and tissue maintenance (24); however, unlike retinol, it is inactive in vision (25) and reproduction (26). The current hypothesis is that retinoic acid is the precursor of, or is itself the systemically active form of retinol. The metabolism of retinol involving these reactions is schematically shown below:



Since the formation of retinoic acid depends upon the availability of retinal as well as the activity of the enzyme which catalyzes its conversion to retinoic acid, it can readily be seen that the utilization of retinal depends upon the relative activities of both retinal reductase and oxidase. Retinal reductase favors the storage of retinol by the conversion of retinal to retinol and subsequently to retinyl ester, while retinal oxidase causes the utilization of retinal through conversion to retinoic acid.

It is apparent that immediately after supplementation with retinyl acetate for five days (Table I), retinal reductase activity is far greater than that of retinal oxidase. It would seem from these observations that the supply of retinal is in excess and that the rate limiting factor is the conversion of retinal to retinoic acid. Interestingly, maintaining these animals on retinol-free diet results in a gradual decrease in the activity of retinal reductase and an increase in the activity of retinal oxidase. The decreased retinal reductase activity would result in a decreased rate of conversion of retinol to retinal. This may explain the build-up of liver retinol during a regimen of retinol-free diet (Table II). The concomitant increase in retinal oxidase activity results in a diminished ratio of retinal reductase to retinal oxidase (Table I). Consequently, a greater proportion of retinal formed will be converted to retinoic acid. This may be an adaptive mechanism for maintaining a normal flow of retinal to retinoic acid in the presence of diminished conversion of retinol to retinal.

Exposure of the animal to a cold environment seems to accelerate these adaptive mechanisms. Both the rate of decrease in retinal reductase and the rate of increase in retinal oxidase are greater in rats exposed to 5 C. After four weeks in the cold, the ratio of retinal reductase to retinal oxidase is less than 1 (Table I), indicating that the rate limiting step for the utilization of retinal is no longer its oxidation to retinoic acid.

It is difficult at this time to assess the physiological significance of these adaptive mechanisms. However, one may be able to explain the data obtained in the present study in the following manner. Because rats are normally reared on a feed which consists of β-carotene as the precursor of retinol, the enzyme retinal reductase is initially adapted to β-carotene and favors storage of retinol. When the rats are placed on retinol-free diet, the rats tend to store less and increase the utilization of retinol. Consequently, it is possible that retinal reductase is lowered with concomitant increase in retinal oxidase activity.

When rats are exposed to cold there is an immediate and sustained increase in metabolic rate (2) which is accompanied by a decreased rate of growth. Johnson and Baumann (3) have shown that these two phenomena have opposing effects on utilization of retinol. Increased metabolic rate results in an increased utilization of retinol, while decreased growth rate is accompanied by decreased utilization. They further show that growth rate is the dominant factor in controlling retinol utilization. The high retinal oxidase activity could presumably be an adaptive response to the elevated metabolic rate as a result of cold exposure. This in itself would result in an increased flow of retinal to retinoic acid. The increased retinoic acid requirement of rats exposed to cold (1) is consistent with this interpretation. Also in line with these observations, Bamji and Sundaresan (27) reported decreased liver storage of retinol in hyperthyroid rats fed retinal, presumably due to increased utilization of retinol.

It is known that thyroid activity in cold exposed rats is increased (28). To offset the increased requirement imposed by cold exposure, the animal's growth rate is diminished. This may be reflected in the decreased retinal reductase activity in cold exposed rats. In consequence, the flow of retinal to retinoic acid would be limited. Experiments are now in progress to assess the role that these enzymes may play in regulating the growth of the animal.

ACKNOWLEDGMENTS

W. T. Matthew gave technical assistance.

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[Received July 15, 1968]

Plasma Lipid and Glucose Levels in the Adrenalectomized Rat Following Triglyceride Ingestion

PAUL H. LENZ, Department of Biology, Farleigh Dixon University, Madison, New Jersey 07904;
ALAN I. FLEISCHMAN and **THOMAS HAYTON,** Atherosclerosis Research Group,
St. Vincent's Hospital, Montclair, New Jersey 07042

ABSTRACT

Adrenalectomy did not significantly alter plasma cholesterol, triglycerides and phospholipids in fasted naturally hypertriglyceridemic rats. Oral administration of cottonseed oil resulted in elevations in plasma cholesterol and phospholipids within 2 hr. Adrenalectomy negated these elevations. Triglycerides rose to an equal extent in both groups at the same time. A secondary rise in plasma glucose was noted at about 7 hr in the control rats; this rise was also negated by adrenalectomy.

INTRODUCTION

Administration of a glucose load has been shown to exert an initial depressant action upon plasma free fatty acids (1,2) followed by a delayed secondary rise (3). This secondary rise has been correlated with increased urinary excretion of 3-methoxy-4-hydroxy mandelic acid (3). Shafrir et al. (4) and Salvador et al. (5) reported epinephrine caused the mobilization of plasma free fatty acids while Barrett (6) noted that changes in lipoproteins could be caused by increases in adrenocortical hormone secretion.

Much of the work to date has been on the interrelationships between free fatty acids, glucose and adrenal hormones. Normally fats are ingested and transported in the circulation as triglycerides. It appeared of interest to examine some interrelationships between triglyceride absorption, plasma lipids, plasma glucose and adrenal hormones. Results of some studies on these interrelationships in the rat are presented in this report.

MATERIALS AND METHODS

In these studies, 60 day old male rats, mean weight 222 SD 6 g, from an inbred hypertriglyceridemic colony of the Long Evans strain were used. They were weaned at 23 days of age and maintained on a commercial rat diet (Purina Fox Chow) and tap water ad lib. Adrenalectomy was done by the method of Ingle and Griffith (7), ensuring that the glands were removed encapsulated. No replacement therapy was given following adrenalectomy. At the start of the experiments all animals were 18 hr postprandial; adrenalectomized animals were 5 days post adrenalectomy. In fat absorption studies, 5 ml of cottonseed oil was given the rats by gavage. In order to obtain sufficient blood for plasma lipids, rats were anesthetized with sodium pentobarbital, 50 mg/kg body weight, at varying times after gavage, and exsanguinated via the dorsal aorta. The blood was collected in tubes containing potassium oxalate (4 mg) and ammonium oxalate (6 mg); the plasma was separated immediately and assayed. Glucose was determined on blood taken from the tail of an additional eight animal cohort studied in four stages to observe the effect of oil gavage in both intact and adrenalectomized animals. Fifty micro liter heparinized capillary tubes were used for sampling.

On each rat plasma cholesterol, triglycerides and phospholipids were determined as previously described (8). Glucose was determined by an ultramicro modification of the glucose oxidase-peroxidase method (9,10,11,-12).

RESULTS

The effects of adrenalectomy upon plasma cholesterol, triglycerides and phospholipids are shown in Table I. The results were obtained on ten rats in each group. No significant differences between the two groups were noted in cholesterol and phospholipids. There was a 28 mg/100 ml increase in plasma triglycerides attributable to adrenalectomy but this increase was not statistically significant due to the large variance.

In Table II are shown the results obtained when 5 ml of cottonseed oil was given by gavage. The results were obtained on five groups of five rats each; one group was killed at each time period. Analysis for changes were performed on the data by the use of appropriate estimates of error and student t-tests (13). In the control rats, plasma phospholipids rose by approximately 16% after 1 hr, $P<0.05$, and then remained essentially constant at the ele-

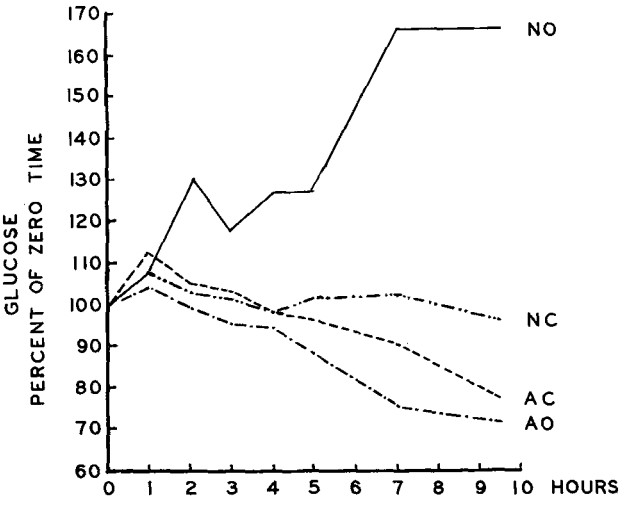

FIG. 1. The effect of triglyceride ingestion upon plasma glucose in adrenalectomized rats. NO, normal rats with oil; NC, normal rats without oil; AC, adrenalectomized rats without oil; and AO, adrenalectomized rats with oil.

vated level for the duration of the experiment. In the adrenalectomized rat no significant change in phospholipids was noted from the fasting level. Plasma cholesterol in the control rats rose by 11% after 1 hr, increased to 24%, $P<0.05$, at 2 hr, and to 45%, $P<0.01$, by 4 hr. Adrenalectomy completely negated these lipid rises in response to a fat challenge. Plasma triglycerides rose in both the control and adrenalectomized animals by approximately 36% and 23%, respectively, between 2 and 3 hr after the fat challenge. By the fourth hour the triglycerides approached the zero time level. Due to the large between-animal variances observed, the apparent differences in the rate and magnitude of triglyceride rise between the adrenalectomized and control rats were not statistically significant.

In Fig. 1 the plasma glucose response to fat loading is depicted. For each response variable and for each animal, normalized values were obtained by computing the ratio of each value to the value at zero time (100%). This treatment materially stabilized the between animal variance observed at each measurement interval. A 29% rise in glucose was observed during the first 2 hr, $P<0.01$, in the control rats. This was followed by a 10% drop, which was not significant, and a subsequent rise

TABLE I

Effect of Adrenalectomy Upon Serum Lipids in 60 Day Old Male Rats

	Control	Adrenalectomized
Body weight, day 0, g	214 ± 4.5[a]	228 ± 9.4
Body weight, day 5, g	221 ± 5.4	206 ± 9.4
Body weight change, g	+7 ± 2.2	−22 ± 3.6
Survival	100%	100%
Phospholipids, mg/100 ml	116 ± 5.8	120 ± 5.8
Cholesterol, mg/100 ml	56 ± 3.0	46 ± 2.7
Triglycerides, mg/100 ml	269 ± 15.0	297 ± 15.0

[a] Mean ± SD.

TABLE II

Plasma Lipid Responses to a Triglyceride Load in Adrenalectomized and Control Rats

	Plasma Lipids (mg/100 ml)	
Time (hr)	Control rats	Adrenalectomized rats
Phospholipids		
0	108 ± 5.8[a]	100 ± 5.8
1	121 ± 6.5[b]	109 ± 5.8
2	125 ± 6.5[b]	106 ± 5.8
3	129 ± 5.8[b]	113 ± 5.8
4	129 ± 6.5[b]	109 ± 6.5
Total cholesterol		
0	45 ± 3.0	46 ± 2.7
1	50 ± 3.0	42 ± 2.7
2	56 ± 2.7[b]	44 ± 2.7
3	55 ± 2.7[b]	51 ± 2.7
4	65 ± 2.7[c]	46 ± 3.0
Triglycerides		
0	267 ± 15	279 ± 15
1	239 ± 17	271 ± 15
2	312 ± 15	364 ± 60
3	363 ± 49	299 ± 15
4	294 ± 37	276 ± 17

[a] ± Standard Deviation of the Mean.

[b] Significant at $P < 0.05$.

[c] Significant at $P < 0.01$.

which at 7 hr was 64% above the value at zero time, $P<0.05$, and remained at this level to at least 9.5 hr. In the absence of a fat load, there was an 8% rise at 1 hr, $P<0.01$, after which the plasma glucose returned to and essentially remained at the zero time level. Adrenalectomy completely negated the glucose rise in response to the fat challenge and actually resulted in a significant drop in plasma glucose of 29% from the fasting level by 9.5 hr, $P<0.01$. In the absence of a fat load, there was an apparent rise of 13% at 1 hr, which was not significant, followed by a significant drop of 23%, $P<0.05$, at 9.5 hr. The response noted in the fat loaded and non-loaded, adrenalectomized animals appeared similar. It would thus appear that adrenalectomy reverses the glucose response to fat load in this rat substrain.

DISCUSSION

Fleischman et al. (14), reported that serum phospholipids in the human increase significantly after 1 hr in response to a triglyceride challenge and remain elevated during the period of elevated serum triglycerides. These results are in agreement with the data reported in the present experiments. Barrett (6) reported that corticosteroids produce increases in cholesterol and phospholipids in the rat. In the present study, the negation by adrenalectomy of this phospholipid rise in response to a fat challenge is suggestive of hormonal control.

It was noted that a fat challenge was associated with a significant but delayed elevation in plasma glucose. Ash et al. (15), reported an increase in plasma glucose following intravenous administration of butyrate and hexanoate. They showed that the glucose elevation was not due to gluconeogenesis from fatty acids. The greatly delayed hyperglycemic effect reported here may be accounted for by the time required for the transport and hydrolysis of the exogenous triglyceride and for the formation of the albumin-free fatty acid complex. The negation by adrenalectomy of the glucose rise following a fat challenge would appear to indicate involvement of adrenal hormones in the production of the hyperglycemic state.

The findings of this study point to the advisability of investigating the interrelationships of adrenal hormones and lipid metabolism.

ACKNOWLEDGMENTS

This work was supported in part by Grant H5905 from the National Heart Institute and Research Grant 9202 from Fairleigh Dickinson University. Horace W. Gerarde of Becton, Dickinson and Company, Rutherford, New Jersey, contributed Unopettes used in the ultramicro determination of blood true glucose.

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[Received July 3, 1968]

Boron Trifluoride as Catalyst to Prepare Methyl Esters From Oils Containing Unusual Acyl Groups

R. KLEIMAN, G. F. SPENCER and F. R. EARLE
Northern Regional Research Laboratory,[1] Peoria, Illinois 61604

ABSTRACT

The procedure of Metcalfe et al. (3) for the preparation of fatty acid methyl esters, using boron trifluoride as catalyst, is shown to be suitable for use with oils containing fatty acids of unusual structures, such as conjugated unsaturation, hydroxyl or epoxy groups, and cyclopropenes in addition to oils with only the common acids. In some cases, boron trifluoride was less destructive to unusual groups than conventional mineral acid catalysts; in others, derivatives were formed that were suitable for quantitation in subsequent gas chromatographic analysis.

INTRODUCTION

Extensive chemical investigation of a variety of vegetable oils requires, for efficiency in operation, a rapid, generally applicable procedure for conversion of the oils to methyl esters. Conventional acid- or base-catalyzed methanolysis involves extended reaction time and recovery of the esters by extraction with solvent. Early procedures employing boron trifluoride (1) or boron trifluoride etherate as catalysts included a solvent extraction step and, if the catalysts were used in too high concentrations, artifacts (polymers or unidentified derivatives) were formed that interfered with subsequent analysis (2). The later method of Metcalfe et al. (3) using 12.5% (w/v) boron trifluoride in methanol eliminated extraction and was found suitable for volatile fatty acids. We have found it to be suitable not only for the usual oils but also for those containing unusual structures such as cyclopropene rings, vicinal hydroxyls, oxirane oxygen, conjugated dienols and conjugated trienes.

A recent report of the Instrumental Techniques Committee (4) indicates that the boron trifluoride method is applicable to the common oils, to dehydrated castor oil and to fats containing isolated *trans* unsaturation, but prohibits its use with some of the structures mentioned above. Results presented here indicate that, if the methyl esters are intended for gas chromatography, the scope of the method may be extended.

METHODS

Ester preparations were catalyzed by hydrochloric acid, sodium methylate, or boron trifluoride. In most preparations catalyzed by mineral acid, oils were refluxed with 5% anhydrous hydrochloric acid or 1% sulfuric acid in methanol for 3 hr, diluted with water, extracted with ether, washed and recovered by removal of the ether under nitrogen on a steam bath. With 0.5N methanolic sodium methylate (5), samples were shaken 3½ hr at room temperature and recovered as above. Boron trifluoride was used essentially as directed by Metcalfe et al. (3) except that Babcock milk test bottles were used for the reactions. Catalyst concentration in the reagent was 5% or 12.5% and reaction times ranged from 2 to 10 min. Hydrochloric acid was used with the Metcalfe technique at the same concentrations and reaction times. Ten millimeters of boron trifluoride etherate (Eastman) was used as catalyst for one sample of soybean oil refluxed for 1 hr. After the heating period, saturated sodium chloride solution was added until the bottle was almost full and the mixture was well shaken. Additional salt solution was added to bring the liquid level into the neck of the bottle. The bottle was centrifuged at 1800 rpm (International Centrifuge head no. 233, carrier no. 362) and the ester layer was drawn off with a narrow tipped pipette.

In one such preparation by this procedure with anhydrous hydrochloric acid-methanol the resulting salt precipitate, from the hydrochloric acid-sodium hydroxide neutralization, was recovered by decantation and washed three times with ether. The salt was redissolved in water and the solution was extracted with ether. The extract was washed with water and the solvent was removed on a steam bath.

All esters were analyzed by GLC on a Burrell K-5 instrument equipped with a thermal conductivity detector (6).

When oil samples of less than 50 mg were esterified, Babcock skim milk test bottles (Kimble no. 530) with a capillary neck were used.

TLC was carried out on plates spread with

[1]No. Utiliz. Res. Dev. Div., ARS, USDA.

a 275 μ layer of Silica Gel G impregnated with boric acid. The plates were developed in a 1 mm "sandwich" open chamber (7), with hexane-ether (70:30) as the developing solvent. The front was allowed to move 15 cm. The spots were visualized by iodine vapor.

Infrared (IR) analyses were performed by use of a Perkin-Elmer Infracord Model 137 with liquid films on sodium chloride disks.

Ultraviolet (UV) absorption was measured with a Beckmann Model DK-2A spectrophotometer in 1 cm silica cells with 95% ethanol as solvent.

Methoxyl groups were determined by the Vieböck and Schwappach method (8).

RESULTS AND DISCUSSION

Substitution of Babcock bottles for volumetric flasks as reaction vessels provides two major advantages: they can be easily centrifuged, thereby causing the esters to separate quickly from the saline solution; and in their slender (or capillary) necks, the esters form in longer columns more easily removable from the aqueous layer.

Use of a capillary-necked flask has been reported (9) but it must be specially made, whereas the Babcock bottle is commercially available.

Esters From Oils With Normal Unsaturation

GLC analyses of esters prepared by the Metcalfe procedure and by the traditional methods show little differences in relative amounts of those acids found in common seed oils. Analysis of *Crambe* oil, in which more than 60% of the acids have chain lengths greater than C_{18}, also gives almost identical results for the two types of ester preparations.

The boron trifluoride-etherate reagent, used with 5 min or 1 hr heating time, produced the previously reported artifact (2) (Table I). The esters prepared from soybean oil, using this catalyst for the longer time, were markedly low in methyl linoleate and the methyl linolenate was completely removed (Table I). Such loss was never observed when boron trifluoride in methanol was used in the amounts and concentrations indicated.

TLC of the reaction products showed only methyl esters except when 5% boron trifluoride reagent was used. In this case, a component was found which corresponded to free acid, indicative of incomplete esterification, but the esters formed were representative of the oil.

When hydrochloric acid was used as catalyst in the Metcalfe procedure, a precipitate of potassium chloride or sodium chloride was formed and yields of ester were low because of occlusion or absorption by the salt precipitate. Esters recovered from the washed precipitate were enriched in polyunsaturates, whereas those recovered by flotation were comparable to esters prepared by the usual methods. The error caused by this effect is negligible for soybean oil but might be serious with other oils.

Esters From Oils With Unusual Acids

UV analysis of the oil of *Maytenus illicifolia* indicated the presence of an aromatic component (10) that, if calculated as benzoic acid (λ_{max} 227 mμ, ε 10,960), constituted 7.9% of the oil. GLC analyses of esters prepared by the hydrochloric acid transesterification procedure with ether extraction failed to show any benzoate. GLC of the esters made by the Metcalfe procedure revealed 2.8% of a component with retention characteristics identical to those of methyl benzoate. No attempt was made to resolve the difference between the UV and GLC results. Acetic acid is also present in *Maytenus* oil (10), but the conditions used for analysis precluded detection of methyl acetate in either preparation.

GLC (Table I) and TLC analysis of the ester preparations, catalyzed by boron trifluoride, from castor and *Lesquerella lescurii* oils showed no alteration of the ricinoleic, densipolic (12-hydroxy-*cis*-9-*cis*-15 octadecadienoic), hydroxypalmitic, and hydroxystearic acids contained in these oils. These results agree with previous work (11) which showed that the hydroxyl group of 2-hydroxymyristic acid was not affected by boron trifluoride.

When boron trifluoride was used as a catalyst for ester preparations of oils containing vernolic acid (*Vernonia anthelmintica* and *Euphorbia lagascae*), almost all of the vernolic acid was converted to a derivative with equivalent chain lengths (12) of 20.2 on Apiezon L columns and 25.1 on LAC-2-R 446 columns (Fig. 1). Only 2–3% of the oil was converted to methyl vernolate (Table I). The mixed esters from the oils had strong IR absorption bands at 2.77 and 9.13μ, the wavelengths associated with hydroxyl and ether groups, respectively (13). Methoxyl analysis of mixed esters from *V. anthelmintica* oil gave 13.9%-OCH_3. The methoxyl analysis supports the assumption that the derivative is the methyl methoxy-hydroxy-oleate. The positions of the substituents are not indicated. The derivative can be used for the quantitative analysis of the esters because its weight per cent can be

TABLE I

Comparison of Esterification Procedures on Some Oils Containing Unusual Fatty Acids

Oil	Catalyst	Normal components by GLC (area %)				Unusual components			
						Equivalent chain length			
		Saturated esters	Monoenoic esters	18:2	18:3	Apiezon L	LAC-2-R 446	%	Identity
Soybean oil	HCl	17	26	52	5	—	—	—	—
	BF_3	17	26	52	5	—	—	—	—
	BF_3 etherate	22	31	24	—	18.1	—	12[a]	—
						18.7	—	8	—
						19.1	20.7	3	—
Maytenus illicifolia[b] Mart.	BF_3	20	42	34	0.1	8.6	11.8	3	Benzoate
						4.0	4.0	0.3	4:0
						8.0	8.0	Trace	8:0
	HCl	20	43	35	1	16.7	—	0.4	Unknown
						19.5	—	0.3	Unknown
Castor oil	BF_3	2	3	5	—	19.6	24.3	90	18:1 OH
	HCl	2	4	5	—	19.6	24.3	89	18:1 OH
Lesquerella lescurii (Gray) S. Wats.	BF_3	12	38	2	15	17.7	23.0	1	16:0 OH
						19.8	24.6	1	18:0 OH
						19.6	24.8	4	18:1 OH
						19.6	25.0	27	18:2 OH
	H_2SO_4	12	35	3	14	17.7	23.0	2	16:0 OH
						19.8	24.6	2	18:0 OH
						19.6	24.8	4	18:1 OH
						19.6	25.0	28	18:2 OH
Vernonia anthelmintica (L.) Willd.	BF_3	6	4	14	0.4	19.2	23.0	3	Vernolate
						20.1	25.1	66	Vernolate derivative
						20.7	29.5	6	Dihydroxyoleate
	$NaOCH_3$	7	5	13	0.1	19.2	23.0	68	Vernolate
						20.7	28.9	7	Dihydroxyoleate
Euphorbia lagascae Spreng.	BF_3	7	23	9	0.3	19.2	23.0	2	Vernolate
						20.1	25.1	58	Vernolate derivative
	$NaOCH_3$	7	22	9	0.4	19.2	23.0	62	Vernolate
Tung oil[c]	BF_3	7	7	7	—	19.3	22.2	79	Conj. 18:3
						19.6	22.6	Trace	
	HCl	7	10	7	—	19.3-19.7	22.2	76	Conj. 18:3
							22.6		
Dimorphetheca sinuata DC.[d]	BF_3	5	13	14	0.2	19.1	21.8	68	Conj. 18:3
						19.9	22.6		
	HCl	5	13	15	0.2	19.5	21.7	66	Conj. 18:3
						19.9	22.6		

[a] Probably the artifact reported by Lough (2).

[b] UV analysis: 7.9% as benzoate.

[c] UV analysis: 83% as α-eleostearic.

[d] UV analysis: 70% as dimorphecolic.

related to the weight per cent of the original methyl vernolate. Under the conditions of analysis used, the response of the derivative seems equal to the response of methyl vernolate. The *V. anthelmintica* oil used in this study contained 7% dihydroxyoleic acid as well as the vernolic acid. Since the GLC analysis of the boron trifluoride-catalyzed esters gave 6% dihydroxyoleic ester, the dihydroxy functional groups were not altered during esterification (Table I).

IR analysis of the boron trifluoride-catalyzed esters from *Sterculia foetida* showed a strong band at 9.92 μ. This band is characteristic of the cyclopropene structure (14). The hydrogen bromide equivalent of the oil at 55 C was 60% (as sterculic acid) and that of the esters was 66%. While the titrations are not in good agreement, these results indicate that the cyclopropenoid functional group in the acids of *Sterculia* oil was undisturbed during the esterification reaction. Conventional acid catalysts altered this functional group, presumably by a reaction analogous to the addition of hydrogen bromide to cyclopropenes (15).

UV analysis of tung oil indicated 84% conjugated triene calculated as α-eleostearic [λ_{max}

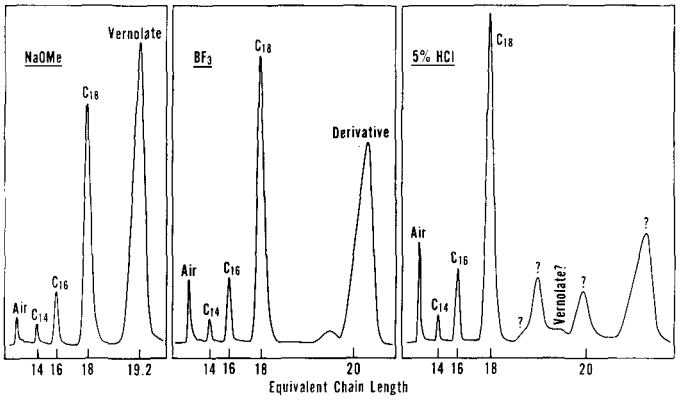

FIG. 1. Gas-liquid chromatography of methyl esters of *Euphorbia lagascae* oil prepared with different catalysts as shown. Column: 125 cm, 1% Apiezon L on Chromosorb G, isothermal at 258C.

270 mμ, ε 47,000 (16)] acid. Esters prepared by refluxing with the hydrochloric acid-methanol reagent have maxima at 258, 268, and 278 mμ while the esters prepared by the boron trifluoride method retained the original chromophore [λ_{max} 261, 270, and 282 mμ (16)]. In addition, the chromatogram resulting from the GLC of the hydrochloric acid esters showed several components in the conjugated triene region while the boron trifluoride esters showed basically one peak (Fig. 2). Quantitation of these chromatograms agreed well: 76% combined conjugated triene in the hydrochloric acid esters and 79% conjugated triene in the boron trifluoride esters. It appears that the prolonged heating required with the hydrochloric acid method, caused isomerization while the boron trifluoride method caused little or none.

The oil of *Dimorphotheca sinuata* contained 70% dimorphecolic acid by UV analysis [λ_{max} 231 mμ, ε 33,600 (17)]. The boron trifluoride-catalyzed ester preparation had UV absorption (λ_{max} 258, 268, and 278 mμ equivalent to 19% calculated as β-eleostearic acid and a broad band at 231 mμ (44% as methyl dimorphecolate). The IR analyses of these esters revealed a strong band at 10.1 μ, indicative of conjugated *trans* unsaturation (18) and showed no evidence of hydroxyl. A band at 9.18 μ indicated the presence of an ether linkage (13). It is asssumed that the absorption at 231 mμ is due to methoxy dienes reported by Powell et al. (19) to be formed from hydroxy dienols in the presence of acid. The methoxyl content of the esters was 14.1%, which is in good agreement with the 14.5% expected of the methoxy diene derivative. The conjugated triene in the esters was presumably formed from dimorphecolic acid by heating under acidic conditions as described by Smith et al. (20). In the GLC analysis, the methoxy diene is converted to conjugated trienes much as acetylated methyl dimorphecolate is converted under the same conditions to conjugated trienes (21).

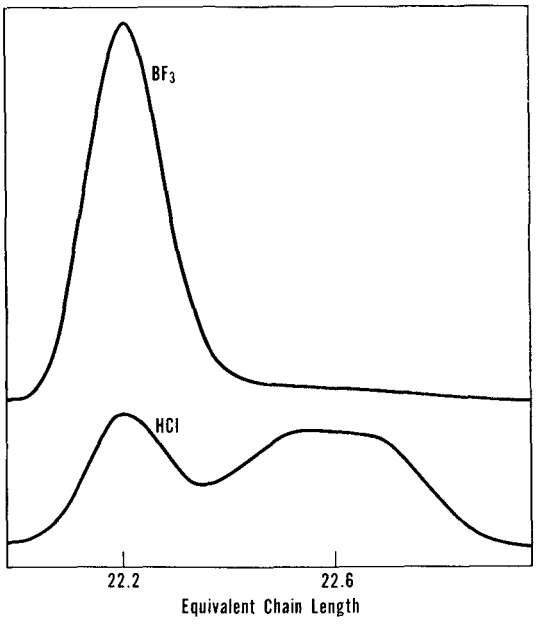

FIG. 2. Conjugated triene region from gas-liquid chromatography of Tung methyl esters. Column: 200 cm, 15% LAC-2-R 446 on Chromosorb W, isothermal at 197C; esterification catalyst as indicated.

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[Received July 11, 1968]

Probable Sources of Plasma Cholesterol During Phosphatide Induced Hypercholesterolemia

SANFORD O. BYERS and **MEYER FRIEDMAN**, Harold Brunn Institute,
Mount Zion Hospital and Medical Center, San Francisco, California 94115

ABSTRACT

Rats in isotopic steady state with respect to 4-^{14}C-cholesterol were infused intravenously with massive amounts of lecithin and also injected once with Na acetate ^{3}H. During the following 8 hr their plasma gained an average of 11.3 mg of cholesterol; the specific activity of ^{14}C-cholesterol fell in plasma while total ^{14}C-cholesterol and ^{3}H activity doubled as compared to controls. The specific activity of ^{14}C-cholesterol diminished in livers of rats receiving lecithin but not in controls. Specific activity of either isotope in cholesterol of intestine, lungs, muscle, skin and brains was the same in control and experimental groups. Total activity of ^{14}C and ^{3}H fell in cholesterol of liver. The results show that plasma accumulation of cholesterol during lecithin infusion derives from both cholesterol pre-existing prior to infusion and from that newly synthesized after the start of infusion and that about one third of this cholesterol of mixed origin is supplied from the liver. The authors speculatively suggest skin as a likely source for most of the remainder, with a small additional contribution from brain.

INTRODUCTION

Massive infusion of phospholipid causes an increase in plasma cholesterol concentration without directly changing the rate of synthesis of cholesterol (1). The increment of plasma cholesterol which is derived from that preexisting in tissues prior to the infusion and the increment derived from newly synthesized sterol (although without increase in the rate of synthesis) is not known. Nor is the relative contribution made by various tissues to the plasma cholesterol known. These questions are of general interest and also of particular interest in connection with therapy of atherosclerosis. It was thought that the method of Wilson (2) for production of an isotopic steady state with regard to 4-^{14}C-cholesterol would suffice to approximate the contribution of cholesterol already existing before phospholipid infusion while pulse injection with ^{3}H-sodium acetate 1 hr after infusion began would allow approximation of the amount of newly synthesized cholesterol. Used together, the two methods yielded a relative estimation of these contributions in the intact rat.

METHODS

Long-Evans strain male rats were used. These weighed approximately 150 g at the time ^{14}C-cholesterol was implanted to begin induction of the steady state. Each animal was anesthetized with ether and a gelatin capsule containing approximately 100 mg of 4-^{14}C-cholesterol (25,000 dpm/mg[1]) was inserted into the subcutaneous tissue through a longitudinal dorsal incision. The animals were maintained on a commercial diet (Simonson Farms) of constant composition and bled from the tail each week under ether anesthesia. A lipid extract of each whole blood sample was assayed for radioactivity as cpm/unit volume by drying at 50 C in a counting vial, dissolving in 10 ml of liquid scintillant and counting in a Packard scintillation counter. Quenching was checked by addition of internal standard and was corrected by the channels ratio method.

At the end of the tenth week an indwelling catheter was inserted into the iliolumbar vein of each rat under ether anesthesia. A 1 ml blood sample (time 0 sample) was withdrawn through the catheter and 1 ml of infusion fluid (lecithin[2] suspension or 0.85% NaCl) injected within 10 sec. The lecithin was given as a 5.2% suspension in 0.85% NaCl, adjusted to pH 7.2 with Na_2HPO_4. Each catheter was connected to a motor-driven syringe delivering 1 ml of infusion fluid per hour. After infusion had continued for 1 hr each rat received through the catheter 0.3 ml of a solution of 32.5 mg (25.0 mc) of ^{3}H sodium acetate (New England Nuclear Lot #163-201-9) dissolved in 8 ml of KH_2PO_4-Na_2HPO_4 buffer, pH 7.2. Infusion was continued at the rate of 1 ml/hr for a total of 8 hr. Blood was obtained through

[1] Cholesterol, regenerated from the dibromide, was dissolved and mixed with isotopic cholesterol. The solution was dried at 50C and the powder dispensed into capsules.

[2] Lecithin suspension was prepared from a mixture of three parts of L-α-(dimyristoyl) lecithin (prepared by C. V. Holland under the supervision of Dr. Jonas Mauruka, Elyria Memorial Hospital, Elyria, Ohio) and one part of essential principal of Lipostabil (1). Liebermann-Burchard positive material in the digitonin precipitate from this suspension, calculated as cholesterol, was 15 mg/100 ml.

the catheter 8 hr after the start of infusion, after which all animals were killed by opening the chest under ether anesthesia and 0.85% NaCl was perfused through the inferior vena cava until liver and intestines were blanched. Tissues were obtained as follows: the entire liver; the entire lungs; the intestine—by cutting between double ligatures immediately caudal to the pylorus and immediately cephalad to the anal sphincter; the entire brain; muscle specimens consisting of portions of the gluteus minimus and rectus femoris muscles; and skin specimens removed from over the rear thigh on the side opposite to that under which the 4-^{14}C-cholesterol pellet had been implanted. The separate tissues were preserved at –10 C.

Plasma was hydrolyzed with alcoholic KOH and extracted with petroleum ether according to the method of Abell et al. (3). The petroleum ether extract was evaporated to dryness on a 50 C hot plate and radioactivity assayed in toluene scintillation solution as before. Cholesterol was quantitatively analyzed in separate samples of these same plasmas by extracting into 20 vol of chloroform-methanol (2:1), partitioning with water (4), evaporating a sample of the chloroform layer to dryness and analysis by the method of Saifer and Kammerer (5).

Tissues were finely divided and extracted with alcohol-acetone (1:1) until the extracts were no longer radioactive. Free cholesterol was precipitated from the combined extracts with digitonin; the clear supernatant was concentrated to one half its volume, hydrolyzed with alcoholic KOH for 50 min at 50 C, acidified and again precipitated with digitonin. The digitonide precipitates were taken just to dryness on a hot plate at 50 C, then dissolved in 1 ml of glacial acetic acid. A sample of the glacial acetic acid solution was removed for analysis by the Saifer and Kammerer method (5). The remainder was dissolved in scintillator solution and radioactivity counted until the count rate error had a standard error of less than 1%. Quenching was checked by addition of ^{3}H and ^{14}C standard, and routinely corrected by the channels ratio method.

Values are reported as means ± SE. The significance of differences between means was estimated by the t-test; values of $P<0.05$ were considered significant.

RESULTS

Attainment of Isotopic Steady State

As reported by Wilson (2), six weeks after implantation of 4-^{14}C-cholesterol capsules the radioactivity per unit volume of whole blood had attained a plateau value characteristic of each individual animal; variation from week to week averaged 9% of each total value, although ranging up to 22% in individual cases. Variation between animals was much greater; the most radioactive blood was more than five times as active as the least. Presumably such differences reflect different rates of absorption of the capsule 4-^{14}C-cholesterol (which may have offered a larger surface in one rat than in another). Prior to infusion, the average specific activity of plasma 4-^{14}C-cholesterol in the entire group of 18 rats (9 later received lecithin) was 845 ± 77 dpm/mg. Since equilibration of liver, plasma and red blood cell free cholesterol occurs rapidly (6,7), in the isotopic steady state this value ought to be also the specific activity of liver cholesterol. This was indeed found to be the case, for when the livers of the 9 rats infused with NaCl were assayed, the specific activity of total cholesterol was found to average 786 ± 104 dpm/mg, that of free cholesterol was found to average 828 ± 90 dpm/mg, and that for ester cholesterol, 855 ± 113 dpm/mg. Since all rats were on a single diet of constant composition, and had attained steady plateau values for blood cholesterol specific activity, they were in a steady state with regard to tissue cholesterol specific activity and concentration. All animals were treated identically until infusion was begun.

Effect of Infusion on Quantity of Cholesterol

Infusion of lecithin for 8 hr increased the average concentration of cholesterol in the plasma of 9 rats from 53 mg/100 ml prior to infusion to 179 mg/100 ml after infusion ($P<0.001$). The 10 rats infused with 0.85% NaCl averaged 62 mg/100 ml prior to infusion and 74 mg/100 ml afterward. This latter difference was not statistically significant. The amount of cholesterol in the entire plasma of each rat was calculated upon the assumption of a plasma volume of 9.5 ml for rats of this weight (366 g), as found by Sharpe et al. (8). The assumption of constant plasma volume, despite infusion of large amounts of saline solutions or of lecithin suspension, is not strictly true. Determination of hematocrit before and after infusion yielded an average value for plasma volume of 54% of the blood volume for each group prior to infusion, changing afterward to 61% and 59% for lecithin and saline groups, respectively. In consequence, the amounts of cholesterol calculated for the post-infusion plasma of the lecithin group are under-

stated by 13% (61/54 × 100) - 100; the corresponding understatement in the saline group is 9%. Prior to infusion the amount of cholesterol in plasma averaged 5.8 ± 0.4 mg and 5.9 ± 0.4 mg of cholesterol for lecithin infused and control rats, respectively. After infusion the respective direct averages were 16.4 ± 1.7 mg and 7.1 ± 0.3 mg of cholesterol. When the amount of cholesterol in the entire plasma of each individual rat prior to infusion was subtracted from the post-infusion value of that same animal, and these individual differences averaged for the two groups, it was found that the plasma of lecithin infused rats gained an average of 11.3 ± 1.7 mg of cholesterol during the 8 hr period. The corresponding value for the control group was 1.1 ± 0.5 mg. The difference, of course, is highly significant.

The cholesterol content of tissues is presented in Table I. The livers of lecithin infused rats average significantly less cholesterol than those of saline infused animals, the difference amounts to 3.8 mg. Other tissues do not differ significantly.

TABLE I
Cholesterol in Tissues After Infusion

Tissue	Saline mg	Lecithin mg
Liver	24.5 ± 0.8[a]	20.7 ± 1.7[a]
Intestine	20.5 ± 0.91	18.4 ± 0.94
Lungs	5.9 ± 0.1	6.4 ± 0.2
Brain (5 rats/group)	23 ± 8	21 ± 10

Values are average mg of cholesterol per entire organ + SE.

[a] These averages are significantly different at the 95% confidence level.

Effect of Infusion on ^{14}C-Cholesterol

The specific activities of plasma and tissue 4-^{14}C-cholesterol are presented in Table II, which shows that during the infusion a significant ($P<0.05$) dilution of specific activity took place in the plasma of the group receiving lecithin.

The variation in absolute values between animals in the same group ranged over 500%, that is, the most radioactive blood contained more than five times as much ^{14}C-cholesterol as did the least. This was apparent before infusion and presumably reflects chiefly different rates of absorption of ^{14}C-cholesterol from the subcutaneous deposit. This variation within a group of course is not referable to any effect of infusion, yet results in large standard errors of the mean which preclude attaching statistical significance to fairly large differences between the means of saline and lecithin groups such as in Table II for plasma, liver and intestine. In the case of plasma and liver, this obscurity can be resolved by using each rat as its own control. With regard to plasma, this was done by subtracting the plasma specific activity of each rat after 8 hr infusion from the pre-infusion value for that same animal. With regard to liver, the same subtraction can be made by taking advantage of the fact that, prior to infusion, the specific activity of ^{14}C-cholesterol in liver is the same as that in plasma. When these computations, using each rat as its own control, were made, the paired differences showed that during 8 hr the specific activity of ^{14}C-cholesterol in plasma diminished by an average of 305 ± 83 dpm/mg in lecithin infused animals and by only 42 ± 23 dpm/mg in those infused with saline. The difference between these respective diminutions is highly significant ($P<0.001$). In the case of liver, when ^{14}C-cholesterol specific activity in liver before infusion (equal to that of pre-infusion plasma) was compared with that of the respective livers after infusion, the specific activity in the livers of lecithin infused rats was found to diminish by an average of 212 ± 56 dpm/mg, while livers of saline infused animals were virtually unaffected, gaining an average of 30 ± 27 dpm/mg. The difference between groups is highly significant ($P<0.001$).

This procedure, using each rat as its own control, cannot be employed for other tissues since the pre-infusion specific activities are unknown. The means for intestine in Table II differ by 15%, but this difference is not statistically significant. The average values for lungs, muscle, skin and brain in each group are similar. The highest specific activity of ^{14}C-cholesterol was that of lung, the lowest that of brain, which was less than one eighth as great.

The radioactivity attributable to ^{14}C-cholesterol in the total volume of plasma (taken as

TABLE II
Specific Activity of Total 4-^{14}C-Cholesterol

Tissue	Saline (10 rats)	Lecithin (9 rats)
Before infusion		
Plasma	811 ± 97	882 ± 133[a]
After infusion		
Plasma	797 ± 109	568 ± 67[a]
Liver	786 ± 104	661 ± 111
Intestine	533 ± 91	626 ± 108
Lungs	1048 ± 100	1020 ± 160
Muscle	623 ± 110	632 ± 141
Skin	410 ± 65	380 ± 72
Brain (5 rats/group)	120 ± 13	121 ± 33

Values are average dpm/mg ± SE.

[a] These averages are significantly different at the 95% confidence level.

TABLE III
Specific Activity of Total ^{3}H-Cholesterol After 8 hr of Infusion[a]

Tissue	Saline (10 rats)	Lecithin (9 rats)
Plasma	486 ± 78	440 ± 76
Liver	515 ± 60	380 ± 43
Intestine	549 ± 35	664 ± 37
Lungs	204 ± 24	208 ± 26
Muscle	56 ± 4	57 ± 7
Skin	106 ± 6	116 ± 10
Brain	12 ± 1	13 ± 1

[a] Each rat received 0.94 mc of ^{3}H-sodium acetate after the first hour of infusion.

Values are average dpm/mg x 10^2 ± SE x 10^2.

None of the averages in the saline group differed significantly from the corresponding averages in the lecithin group at the 95% confidence level.

9.5 ml, see Ref. 8) averaged 4579 ± 560 (dpm ± SE) and 4531 ± 950 in the saline and lecithin groups, respectively, before infusion; the corresponding values after infusion were 5434 ± 703 and 9509 ± 1586. These two latter values differ at the 95% confidence level, while the increase in the lecithin group is significant at the 99% level. By either index, then, lecithin infusion is seen to approximately double the ^{14}C-cholesterol activity in plasma. The amounts of ^{14}C-cholesterol radioactivity before and after infusion in those other tissues where this was determined were, for saline and lecithin infusions respectively: liver, 19,200 ± 2700 (dpm ± SE) and 14,000 ± 2800; intestine, 10,900 ± 1700 and 11,200 ± 1400; lungs, 6706 ± 616 and 6583 ± 1127; brain, 2699 ± 344 and 2622 ± 1000. None of these averages show statistically significant differences between saline and lecithin infused groups, though in the case of liver the difference is great enough to suggest the possibility of attaining significance if more rats were used.

Contribution of ^{3}H to Cholesterol

The specific activities of plasma and tissue tritiated cholesterol are presented in Table III. No significantly different specific activity in the lecithin infused animals is seen in comparison with that of the saline infused group. The lower value for specific activity in the livers of lecithin infused animals is suggestive in that it approaches, but does not reach, statistical significance.

The total radioactivity attributable to tritiated cholesterol in plasma and tissues is presented in Table IV. The plasma of lecithin treated animals contained more than twice as much ^{3}H-cholesterol radioactivity as did control plasma. Conversely, the livers of lecithin treated rats contained significantly less ^{3}H-cholesterol activity than did those of controls.

TABLE IV
Total Radioactivity of ^{3}H-Cholesterol After 8 hr of Infusion

Tissue	Saline (10 rats)	Lecithin (9 rats)
Plasma	36 ± 4[a]	76 ± 13[a]
Liver	124 ± 14[a]	77 ± 8[a]
Intestine	109 ± 10	118 ± 11
Lungs	13 ± 1	13 ± 1
Brain (5 rats/group)	3 ± 1	3 ± 1

Values are dpm x 10^4 per entire organ ± SE x 10^4. Plasma total volume is taken as 9.5 ml (8).

[a] The value in the column marked Saline is significantly different from the corresponding value in the column marked Lecithin at the 99% confidence level.

DISCUSSION

In a previous study (1) we reported that the excess cholesterol accumulating in the plasma of the rat infused with lecithin did not result from a primary increase in the rate of cholesterol synthesis, that is, the fraction of administered ^{14}C-acetate incorporated into cholesterol remained the same in experimental and control groups after 8 hr infusion, as did expired $^{14}CO_2$ and excreted acetyl-1-^{14}C-*p*-aminobenzoic acid. However, in this previous study the organ or tissue provenance of such plasma excess cholesterol was not determined, nor was it determined whether this excess cholesterol was pre-existing or newly synthesized (albeit without increase in the rate of such synthesis).

In the present study we again observed similarity in the rate of incorporation of acetate into cholesterol; in this instance after 1 hr infusion. Thus, the ^{3}H-cholesterol radioactivity found in the entire plasma volume plus that in the liver and intestine (Table IV) was 271 ± 24 x 10^4 dpm for the lecithin group and 269 ± 22 x 10^4 dpm for the saline controls.

The present study indicates that part of the plasma cholesterol was derived from that newly synthesized in the liver and that such new (^{3}H-labeled) cholesterol, although its actual weight is not known, was twice as abundant in the plasma of lecithin infused rats as in that of controls. In addition, some of the plasma cholesterol evidently came from pre-existing (^{14}C-labeled) cholesterol present in the animal's body prior to infusion (see Results section), since the total amount of ^{14}C label also was increased in plasma cholesterol.

Examination of the ratios ^{14}C/^{3}H, obtained by dividing each value in Table II by the corresponding value in Table III, shows differences in plasma and liver of the lecithin group. The ratio is 568/440 = 1.29 in plasma and 661/380 = 1.74 in liver for this group, while the corresponding values for the saline group

are 797/486 = 1.64 and 786/515 = 1.52. Saline infusion is seen not to disturb the equilibrium between plasma and liver which existed prior to infusion, whereas this is disturbed by lecithin infusion. The changes in specific activity which alter the $^{14}C/^{3}H$ ratios suggest differences in rate of transport of old and new cholesterol. Thus, the ratio of 1.29 in plasma results chiefly from the lower specific activity of ^{14}C-cholesterol while the ratio of 1.74 in liver results chiefly from a low specific activity of ^{3}H-cholesterol. This suggests newly synthesized sterol is preferentially removed from liver to plasma under the influence of lecithin.

While cholesterol labeled with either isotope was increasing in the plasma of rats infused with lecithin, it was diminishing in the livers of those same rats, whether measured by the ^{3}H content, or by weight. Content of ^{14}C-cholesterol also diminished but the amount of diminution did not attain statistical significance. However, the deficit of liver cholesterol was not sufficient—indeed, in our previous work (1), we did not notice this deficit—to account for the increment in plasma cholesterol. Thus the livers of lecithin rats lost an average of less than 4 mg of cholesterol as compared with controls while the plasma gained over 11 mg. The relative loss of ^{3}H-cholesterol from the liver is significant statistically and is more than sufficient to account for the excess ^{3}H-cholesterol in plasma of lecithin treated (Table IV).

From the above, it seems likely that most cholesterol added to the plasma of lecithin treated rats was pre-existing and came from tissues other than liver. In this connection, it should be recalled that lecithin infusions will elevate the blood cholesterol level of the rat even when the liver is excluded from the circulation (9). An indication of which tissues are most likely sources can be derived from consideration of the reason for the drop in specific activity of plasma ^{14}C-cholesterol during lecithin infusion. Possible sources of the increments of ^{14}C-cholesterol (at various specific activities in various organs) entering the plasma can be indicated roughly if assumption is made that such increments simply enter plasma and remain in plasma. It must be emphasized that this assumption is for purposes of rough calculation only and does not deny the actual exchange of cholesterol molecules between fixed tissues, body fluids and erythrocytes. In the isotopic steady state, before the start of infusion, each tissue maintains its ^{14}C-cholesterol at a specific activity characteristic of the tissue, being in balance between new synthesis and contributions to and from plasma. Assume then that: the 5.8 mg of plasma ^{14}C-cholesterol at specific activity 882 dpm/mg found in plasma before the start of infusion remains in the plasma, as does the 1.4 mg of Liebermann-Burchard positive material at specific activity of 0 dpm/mg which is introduced with the 9 ml of lecithin infusion; and that the 3.8 mg of liver cholesterol deficit also remains in plasma at the end of infusion after entering at specific activity 661 dpm/mg (the final specific activity of ^{14}C-cholesterol in livers of lecithin treated rats). Since the final amount of cholesterol in plasma is 16.4 mg at specific activity of 568 dpm/mg, then 16.4 – (5.8 + 1.4 + 3.8) = 5.4 mg of ^{14}C-cholesterol from tissues other than liver remains in plasma after having entered at a specific activity which satisfies the relation, S.A. =

$$\frac{(16.4 \times 568)-(5.8 \times 882 + 1.4 \times 0 + 3.8 \times 661)}{5.4} = 313 \text{ dpm/mg}$$

Of the tissues measured, this specific activity is closest to that of skin, 380 dpm/mg.

Chevallier and Giraud (10) have found 23% of the total brain cholesterol of the adult rat to be exchangeable with plasma cholesterol. Chevallier and Petit (11) have shown that although the rate of exchange is slow in anatomical locations where the proportion of exchangeable cholesterol in adult rat brain is high, a rapid exchange takes place where the quantity of exchangeable cholesterol is small. These latter authors suggest that in one of the structures adjacent to myelinated fibers all the cholesterol is totally exchangeable. Kabara et al. (12) have found 18% loss of brain cholesterol in mice within 4 hr after being given a psychomotor stimulant; this loss reached a maximum of 20% at 24 hr. It therefore seems permissible to introduce the further assumption of a contribution to plasma of 1 mg of cholesterol from brain at specific activity 121 dpm/mg. When calculated as above, the specific activity at which the remaining 4.4 mg of cholesterol enters the plasma then becomes 381 dpm/mg; this is the same specific activity as skin cholesterol. Of course, such calculations are speculative, at most, suggestive. However, in the isotopic steady state most tissues have specific activities similar to that of blood (10) so that, once again, only skin and brain carry ^{14}C-cholesterol at sufficiently low specific activity to be supposed to bring about the observed drop in plasma ^{14}C-cholesterol specific activity.

Consideration of the amount of cholesterol in rat tissues reinforces this speculative conclusion. Most rat tissues contain little choles-

terol. Those containing most are skin and muscle (13), which account for over 80% of the cholesterol in rat carcass minus brain. Since the cholesterol content of the entire carcass minus brain is about 1.6 mg/g carcass weight, a shift of 2% into plasma would be more than enough to account for the increment of plasma cholesterol over and above that furnished by liver. A shift of 3% of skin cholesterol into plasma would suffice and, of course would not entail any change of the specific activity of cholesterol in skin.

The sources of ^{3}H-cholesterol are subject to a similar speculation. Thus lecithin infusion doubled the amount of ^{3}H-cholesterol activity in plasma just as it approximately doubled the amount of ^{14}C-cholesterol activity and, at the the same time, lecithin diminished the total ^{3}H-cholesterol activity in liver. However the specific activity of ^{3}H-cholesterol in plasma did not diminish, as compared with controls. This implies that under the influence of lecithin ^{3}H-cholesterol was furnished to plasma from areas of relatively high specific activity, in contrast to the case of ^{14}C-cholesterol. Such high specific activity areas are most probably liver and intestine, which are known to be the tissues most active in synthesis of cholesterol from intravenously injected isotopically labeled acetate (6).

In conclusion, the results suggest liver as one of the organs contributing a considerable amount of both the newly synthesized and preformed cholesterol which accumulates in plasma after lecithin infusion in the rat; however, the bulk of this cholesterol originates elsewhere. Speculatively, the most likely additional sources are suggested to be skin and possibly brain.

ACKNOWLEDGMENT

Technical assistance was provided by W. Hayashi. D. Henderson, M. King and C. Omoto. Aided by grant from the National Institutes of Health, National Heart Institute.

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[Received May 9, 1968]

α-Oxidation of 2-Hydroxy Tetracosanoate in the Rat

KENNETH LIPPEL and **JAMES F. MEAD,** Department of Biological Chemistry, UCLA School of Medicine and Laboratory of Nuclear Medicine and Radiation Biology, Los Angeles, California 90024

ABSTRACT

Four hours after oral administration of uniformly tritiated 2-hydroxy tetracosanoate, most of the dose was recovered in the lipids of the digestive organs and liver of the rat. In contrast to the unchanged substrate detected in the lipids of the stomach tissue, the radioactivity recovered from the cells of the intestine and from the liver was predominantly in saturated fatty acid moieties of complex lipids and the small per cent of recovered hydroxy tetracosanoate was also incorporated into these lipids.

INTRODUCTION

Hydroxy fatty acids are normally found in brain and other tissues as amide-linked components of cerebrosides (1). Cerebrosides are degraded to ceramides (2–4) which may be hydrolyzed by liver, brain or kidney ceramidase to sphingosine and fatty acids (5), but the presence of ceramidase in the intestine has not been demonstrated. The oxidation of the straight chain 2-hydroxy fatty acids by plant particulate fractions has been reported (6) and the in vivo degradation of these acids has also been studied in yeast (7) but no reports concerning their metabolism in vivo in mammalian systems have been published. The present investigation was undertaken to study the in vivo metabolism of 2-hydroxy tetracosanoate, a major component of brain cerebrosides (8).

MATERIALS AND METHODS

Lipid Samples and Reagents

Fatty acids, methyl esters, phosphatidylcholine, cerebrosides and hydroxyceramide were purchased from Applied Science Laboratories, State College, Pa., and the Hormel Institute, Austin, Minn. Cholesteryl oleate and mono-, di- and tripalmitin were bought from Supelco Inc., Bellefonte, Pa. Phosphatidyl ethanolamine was the product of Mann Research Labs, New York, N. Y. Solvents and chemicals were analytical reagent grade from J. T. Baker Co., Phillipsburg, N. J. and *n*-pentane was bought from Phillips Petroleum Co., Bartlesville, Okla. The silicic acid used for column chromatography was Baker analytical reagent grade and the silica gel G used for TLC was obtained from E. Merck A.G., Darmstadt, Germany. Hydroxide of Hyamine 10-X [*p*-diisobutyl cresoxyethoxyethyl-dimethylbenzyl-ammonium Hydroxide] was purchased from Packard Instrument Co., Downers Grove, Ill. and Tween-20 (polyoxyethylene sorbitan monolaurate) was the product of Atlas Chemical Industries, Wilmington, Del.

Substrate Preparation and Administration

Uniformly titrated 2-hydroxy tetracosanoate (A. J. Fulco of this laboratory) was purified by silicic acid chromatography of the methyl ester and recrystallization of the free acid from petroleum ether (7). A clear suspension of this acid (0.85 ml in distilled water and containing 6.2 $\times 10^6$ cpm/ml (1.6×10^9 dmp/μmole) and 1% Tween-20) was administered orally to 24-hr fasted rats (Carworth Farms, Rockland, N. Y.) under light ether anesthesia using a blunt needle and syringe. After 4 hr, the organs were removed, weighed and homogenized for 1 min in a Waring blender in 20 volumes of chloroform-methanol (2:1 v/v). After filtration, the extracts were washed free of non-lipid contaminants (9).

Thin-Layer Chromatography

Radioactive compounds were separated on 20 × 20 cm glass plates coated with a 250 micron thick layer of silica gel G. The plates were activated at 110 C for 1 hr prior to use and developed in various solvent systems. Successive 1 cm segments were scraped from the plates and assayed for radioactivity. Reference compounds were spotted on each plate and visualized by spraying with an alkaline solution of bromophenol blue.

Aliquots of the stomach tissue, small intestine tissue and liver lipid extracts were analyzed on plates developed 10 cm past the origin with benzene-chloroform-methanol-acetic acid (80:20:20:1, v/v). The polar lipid segment (R_f 0–0.15), the middle segment (R_f 0.15–0.55) and the non-polar segment (R_f 0.55–1.0) were eluted, subjected to methanolysis by heating with 4% HCl in methanol (7), and analyzed for unsubstituted (R_f 0.8–0.9) and hydroxy (R_f 0.40–0.55) methyl esters on plates developed 10 cm in petroleum ether-ether (1:1 v/v). The stomach lipid extract, after treatment with diazomethane (10) was similarly analyzed for unsubstituted and hydroxy esters.

TABLE I

R_f Values of Standard Compounds in Several Solvent Systems

Compound	Petroleum ether-ether (1:1 v/v)	Benzene-$CHCL_3$-MeOH-acetic acid (80:20:20:1v/v)	(1) Petroleum ether-ether (80:20 v/v) (2) *n*-Propanol-acetic acid (100:1 v/v)
Stearic acid		0.70 – 0.80	
Monopalmitin		0.47 – 0.60	
Dipalmitin		0.77 – 0.89	
Tripalmitin		0.87 – 0.97	
Cholesterol oleate		0.87 – 0.97	
2-hydroxytetracosanoic acid	0.00	0.23 – 0.40	
2-hydroxytetracosanoate methyl ester	0.40 – 0.55	.....	0.80 – 0.83
Hydroxy ceramide		0.40 – 0.50	
Cerebroside		0.20 – 0.28	
C_{16} to C_{24} unsubstituted acid methyl esters	0.80 – 0.90	0.9	0.87 – 0.93
Phosphatidyl choline		0.00 – 0.03	
Phosphatidyl ethanolamine		0.05 – 0.10	
Mercuric acetate adduct of oleic acid			0.63 – 0.77
Mercuric acetate adduct of linoleic acid			0.33 – 0.50
Mercuric acetate adduct of linolenic acid			0.17 – 0.22
Mercuric acetate adduct of arachidonic acid			0.01 – 0.03

Silicic Acid Column Chromatography

A separate aliquot of the total intestinal lipid extract was treated with HCl-methanol and pipetted onto a 15 g silicic acid column (2.5 cm i.d. × 7.6 cm high) and eluted with 150 ml fractions of 1% to 10% ether in pentane, then with ether, and finally with methanol. One milliliter aliquots of each fraction were assayed for radioactivity. The 1–5% and 6–10% ether in pentane eluates were pooled separately. Each pool and the ether and methanol eluates were analyzed on thin layer plates for unsubstituted and hydroxy esters as described above.

Gas-Liquid Chromatography

In a separate experiment, the intestinal lipids were separated as described previously. The unsubstituted methyl esters were eluted from a silicic acid column with 3% ether in pentane and analyzed on a Cary-Loenco gas chromatograph with ionization chamber and dual pen recorder using 4 ft × ¼ in. stainless steel columns coated with either 10% DEGS on 60/80 mesh Chromosorb W treated with hexamethyldisilizane and operated at 240 C using helium at 20 lb/in.^{2} gage pressure. The retention times of standard methyl esters were determined on each column and the per cent of the unknown methyl esters was estimated by comparing the areas under the radioactive peaks.

TABLE II

Tissue Distribution Radioactivity Following Administration of Uniformly Titrated 2-^{3}H-Hydroxytetracosanoate

Organ	Wet Weight, g	cpm/Organ	Per cent of administered dose
Small Intestine + contents	7.0	3.1×10^6	60.0
Stomach + contents	2.5	2.1×10^5	3.9
Large intestine + contents	7.0	6.0×10^5	11.2
Liver	8.5	1.4×10^5	2.5
Kidneys	2.0	8.8×10^3	0.2
Spleen	1.5	6.5×10^3	0.1
Heart	1.0	3.6×10^3	<0.1
Lungs + esophagus	2.0	5.2×10^3	0.1
Mesenteric fat	8.0	1.1×10^4	0.2
		Total	78.1

Mercuric Acetate Adducts of Unsaturated Fatty Acid Methyl Esters

Aliquots of either unsubstituted intestinal methyl esters eluted from the silicic acid column with 1–5% ether in pentane or of reference unsaturated methyl esters were allowed to react with mercuric acetate reagent (11,12). The extracted products were fractionated on a plate developed 15 cm in petroleum ether-ether (80:20 v/v). After air drying, the plate was redeveloped 15 cm in the same direction with *n*-propanol-acetic acid (100:1 v/v). The R_f values of reference compounds in the various solvent systems are summarized in Table I.

Other Methods

Radioactivity was assayed on a Packard 3003 Scintillation counter using either toluene or naphthalene-dioxane solutions (13); counting efficiency was 80%. Protein was determined colorimetrically at 640 mμ/ by the biuret reaction (14).

RESULTS

Distribution of Administered Radioactivity

Most of the recovered radioactivity was found in the lipid extract of the small intestine with smaller amounts in the extracts of large intestine, stomach and liver. The other organs examined contained little radioactivity (Table II).

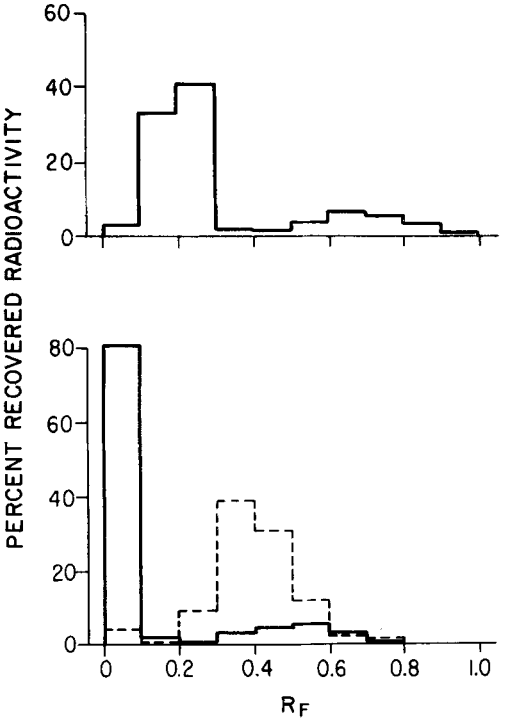

FIG. 1. TLC distribution of radioactivity in lipid extract of stomach. Top half of figure: Plate developed 10 cm with benzene-chloroform-methanol-acetic acid (80:20:20:1 v/v). Bottom half of figure: Plate developed before (solid line) and after (dotted line) treatment of lipid with diazomethane with petroleum ether-ether (1:1 v/v).

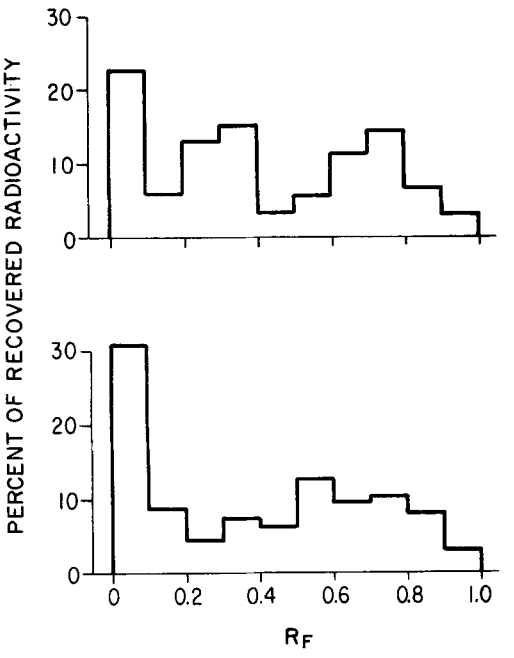

FIG. 2 TLC distribution of radioactivity contained in total lipid extracts of small intestine (upper half of figure) and liver (lower half of figure). Plate developed 10 cm in benzene-chloroform-methanol-acetic acid (80:20:20:1 v/v).

TLC Analysis of the Lipid Extracts of Stomach, Intestine and Liver

A major radioactive peak was detected when the stomach lipids were analyzed using benzene-chloroform-methanol-acetic acid (80:20:20:1) solvent system. After treatment with diazomethane and TLC analysis with petroleum ether-ether (1:1 v/v) the R_f of the major radioactive peak changed from an R_f of 0-0.1, corresponding to the R_f of the 2-hydroxy-tetracosanoic acid to an R_f of 0.4 ± 0.1 corresponding to the R_f of methyl,2-hydroxy tetracosanoate (Fig. 1 and Table I).

Several radioactive zones were detected in the lipid extracts of both the intestine and the liver. The polar lipid segment contained 25–45% of the recovered radioactivity, the middle segment 10–30%, and the remaining radioactivity was detected in the nonpolar segment of the plate (Fig. 2). After methanolysis, most of the radioactivity from each segment was detected at an R_f (0.73–0.90) corresponding to the R_f of unsubstituted methyl esters. Less than 10% of the recovered radioactivity was detected at the R_f (0.5) corresponding to that of methyl,2-hydroxy tetracosanoate. An additional radioactive peak (R_f 0.65) was detected in the radioactivity eluted from the middle segment of the plate containing the intestinal lipid extract (Fig. 3).

Analysis of the Intestinal Fatty Acid Methyl Esters

An aliquot of the intestinal lipid extract was treated with HCl-methanol and analyzed on a silicic acid column. In agreement with the results obtained by thin-layer analysis, 71.4% of the recovered radioactivity was eluted in the unsubstituted ester fraction with 1–5% ether in pentane while only 8.8% of the radioactivity was eluted in the hydroxy ester fraction with 6–10% ether in pentane. The remaining radioactivity was eluted from the column with ether (14.3%) and methanol (5.4%). The eluted fractions were tested for homogeneity on thin-layer plates developed in petroleum ether-ether (1:1 v/v). More than 85% of the recovered radioactivity of the pooled 1–5% ether in pentane eluate was detected in a peak (R_f 0.9) corresponding in R_f to that of reference unsubstituted methyl esters while the remaining radioactivity was detected at R_f 0.5 corresponding

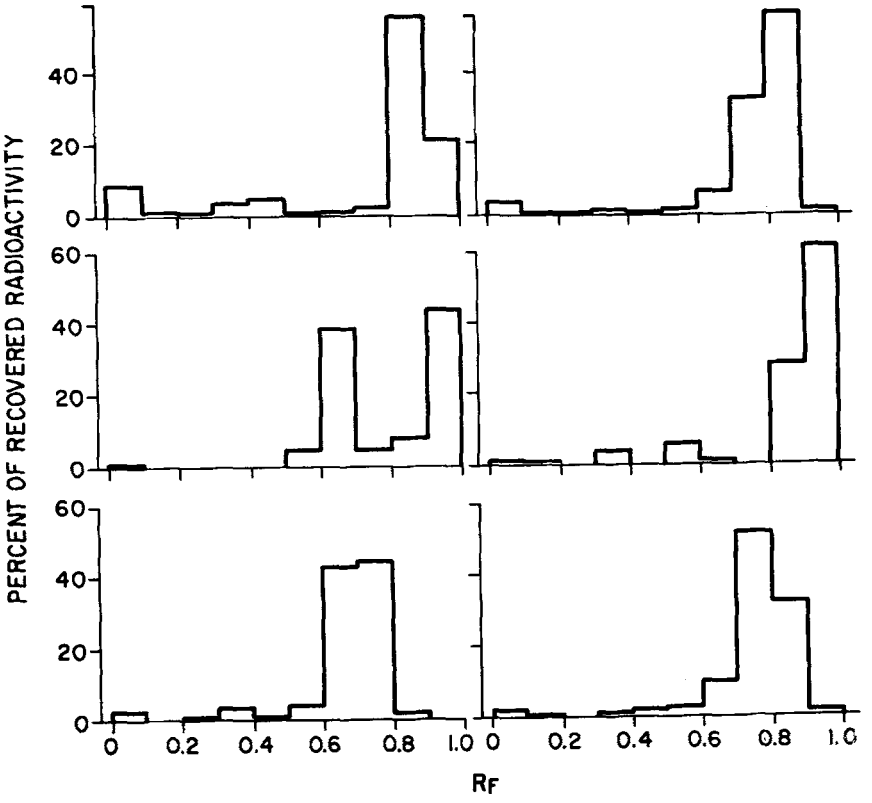

FIG. 3. TLC distribution of radioactivity contained in intestinal (left side of figure) and liver (right side of figure) methyl esters derived from the polar (upper quadrant), middle (center quadrant), and nonpolar (lower quadrant) segments of thin-layer plates developed in benzene-chloroform-methanol-acetic acid (80:20:20:1 v/v) solvent system. Plate developed 10 cm in petroleum ether-ether (1:1 v/v).

TABLE III

GLC Relative Retention Times and Radioactivity of Fatty Acid Methyl Esters From Intestine[a]

Acid methyl ester carbon number	Standard	Apiezon L	3% Ether eluate	Standard	DEGS	3% Ether eluate
15:0			0.32 (3)			0.38 (2)
16:0	0.47		0.50 (4)	0.52		0.55 (3)
17:0	0.68		0.66 (26)	0.72		0.71 (28)
18:0	1.00	1.00	1.00 (9)	1.00	1.00	1.00 (4)
19:0	1.45		1.44 (18)	1.36		1.48 (22)
20:0	1.96	2.17	2.00 (trace)	1.90	2.10	(trace)
21:0	3.08		2.96 (trace)	2.62		(trace)
22:0	4.49	4.64	4.35 (trace)	3.60	3.92	(trace)
23:0	6.46		6.14 (40)	4.84		4.33 (41)

[a]Above retention times are relative to retention times for methyl stearate of 10.1 and 10.3 min on Apiezon and 5.0 and 5.6 min on DEGS columns respectively. Figures in parentheses are relative percentages of areas under radioactive peaks.

to the R_f value of methyl,2-hydroxy tetracosanoate. In contrast, approximately 48% of the recovered radioactivity in the pooled 6–10% ether in pentane eluate was detected at R_f (0.3–0.5) and the remaining radioactivity was located in a second peak (R_f 0.5–0.8) intermediate in polarity between the hydroxy and unsubstituted esters (Table I). Although the R_f value of this unknown peak corresponded to the R_f value of dimethyl acetals in this solvent system (15), the dimethyl acetals would have been eluted from the silicic acid column with 3% ether in pentane (16). Unidentified polar components containing 54% and 70% of the recovered radioactivity were detected at R_f 0–0.2 when the ether and methanol eluates were similarly analyzed. These results are illustrated in Figure 4.

The unsubstituted esters eluted from the silicic acid column were analyzed for degree of

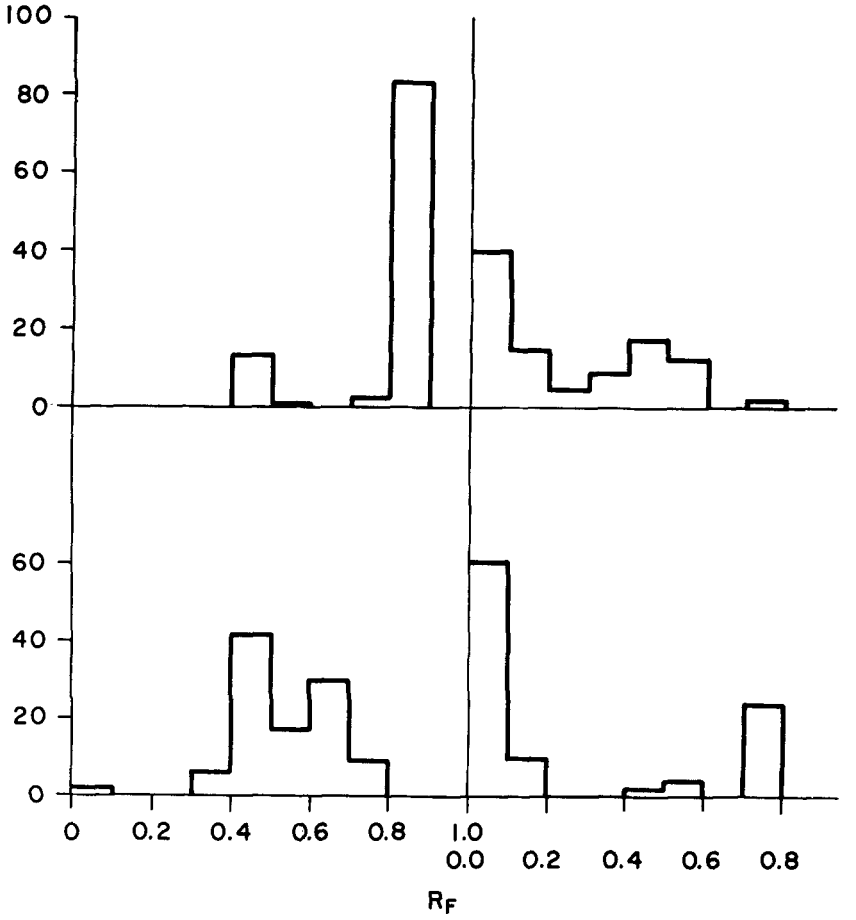

FIG. 4. TLC distribution of radioactivity of intestinal methyl esters eluted from a silicic acid column with 1–5% ether in pentane (upper left), 6–10% ether in pentane (lower left), ether (upper right) and methanol (lower right). Plate developed 10 cm in petroleum ether-ether (1:1 v/v).

unsaturation. Most of the radioactivity was detected in a single peak at R_f 0.9 corresponding to that of saturated methyl esters. Less than 10% of the recovered radioactivity was detected at an R_f corresponding to that of oleic acid adduct and only traces of radioactivity were detected at R_f values corresponding to those of mercuric acetate adducts of polyunsaturated acids (Table I).

The unsubstituted methyl esters eluted from the silicic acid column were analyzed on the GLC column. The retention times of the radioactive peaks corresponded to those of both odd and even carbon number unsubstituted methyl ester standards. A large proportion of the recovered radioactivity was contained in the tricosanoic acid peak and in other peaks corresponding to odd chain methyl esters. The results are summarized in Table III.

DISCUSSION

Previously, α-oxidation of the 2-hydroxy fatty acids has been studied only in the brain (17,18) since these acids occur predominantly in this organ as amide-linked components of cerebrosides. In the present investigation, extensive metabolism of orally administered 2-hydroxy tetracosanoic acid in the rat intestine was noted. Although it cannot be stated with assurance that this metabolism was not due to the intestinal flora, the finding that very little hydroxy acid appeared in the small intestine extracts and that decarboxylation occurred prior to entry into the large intestine would indicate

that this was the case. Based on the elution patterns from silicic acid and gas chromatographic columns and on the R_f values on this layer plates, the saturated long chain fatty acids are apparently the major metabolic products. The finding of tricosanoic acid as a major product indicates that 2-hydroxy tetracosanoate is initially α-oxidized and that the saturated fatty acids are then presumably formed from tricosanoic acid by stepwise β oxidation. Although intestinal ceramidase activity has not yet been demonstrated, ingested amide-linked hydroxy fatty acids after cleavage of the amide bond, initial α-oxidation to the next lower unsubstituted acid, and subsequent β-oxidation, may be one source of the small amounts of odd chain fatty acids detected in the lipid extracts of various tissues. We have also noted that 2-hydroxystearate is more rapidly decarboxylated by heart, liver and kidney homogenates than by brain homogenates (19) but have not yet determined if the release of carbon dioxide occurs by α-oxidation or some other mechanism. However, the present results together with the finding that phytanic acid is α-oxidized after intravenous injection (20) indicate that α-oxidation occurs in tissues other than the brain. More than one α-oxidation enzyme system apparently exists since the phytanic acid oxidizing system is localized in rat liver mitochondria (21) whereas the 2-hydroxy long chain fatty acid oxidizing system is located predominantly in the microsomes (17). In addition to radioactive peaks corresponding in R_f (0.8) to that of unsubstituted fatty acids, small radioactive peaks corresponding in R_f (0.4) to that of methyl,2-hydroxytetracosanoate were detected (upper and lower quadrants, left side of Fig. 3) when the intestinal lipids from the polar segment and from the nonpolar segment of the thin-layer plate (upper half of Fig. 2) were analyzed for unsubstituted and hydroxy esters. Small amounts of hydroxy tetracosanoate were apparently incorporated into nonpolar and polar lipids (Table I). Although ester-linked 2-hydroxy acids of 12 carbon chain length have been reported in bacteria (22), longer chain 2-hydroxy fatty acids have previously been reported in mammalian brain only in amide linkage as components of cerebrosides.

Another point of interest stems from the finding that the products of β-oxidation were stabilized by incorporation into various lipids. Traditionally, the products of β-oxidation have been thought of as undergoing further oxidation without release from the enzyme. However, more recent evidence (cf. 23) has indicated that at least those fatty acids with chain-lengths of 16 carbons or greater can be released and even elongated. In the present experiments, all the odd-chain fatty acids, which could only have arisen by degradation, were present in the intestinal lipids.

Whether the α-oxidation reaction normally takes place with unsubstituted fatty acids in these tissues will be the subject of another investigation.

ACKNOWLEDGMENT

Supported by Grant HE 05306 from National Institutes of Health, U.S.P.H.S.; U.S.P.H.S. Research Career Award GM-A679,177; supported in part by Contract AT(04-1) Gen-12 between the Atomic Energy Commission and the University of California.

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[Received July 26, 1968]

A Rapid Microozonolysis-GLC Procedure for Locating Unsaturation in Olefinic Acids, Including Trienes and Tetraenes[1]

R. KLEIMAN, G. F. SPENCER, F. R. EARLE and I. A. WOLFF
Northern Regional Research Laboratory,[2] Peoria, Illinois 61604

ABSTRACT

Increased versatility has been achieved in the identification of unknown olefinic fatty acids by ozonolysis. The method has been applied to purified methyl esters containing up to four double bonds. Aldehydic fragments, obtained from esters by the Stein-Nicolaides procedure (2), were determined by GLC on two columns of different polarity. Equivalent chain lengths of each fragment on the two columns provide identification. For monoenoic esters the location of the double bond is clearly indicated by the aldehyde and aldehyde-ester fragments. Dienes are identified by the aldehyde and aldehyde-ester fragments when the original chain length of the ester is known; the dialdehyde fragment provides confirmatory evidence. Trienes and tetraenes are analyzed by interrupting the ozonolysis at various times, thereby producing unsaturated, as well as saturated, aldehydes and aldehyde-esters. Unsaturated fragments locate the central or interior double bonds.

INTRODUCTION

In the GLC analysis of methyl esters prepared from vegetable oils, components are usually identified as to chain length and number of double bonds. Such identification is not specific unless supported by rigorous characterization by chemical or physical means. Ozonolysis has become widely used in determining the location of double bonds in fatty acids. The method reported here can be used without repeated ozonolysis of known materials in order to identify components and is of general applicability to mono- and polyenoic fatty acids, even those containing double bonds more widely separated than the common methylene interruption. The method was used with esters having both *cis* and *trans* unsaturation.

METHODS

A 0.2% solution of purified methyl esters (1 to 10 mg) was prepared in dichloromethane (Matheson Coleman and Bell, superior grade). The solution was cooled in an acetone-dry ice bath and the ozone-oxygen mixture from a Bonner (1) ozone generator was bubbled through at 30 ml/min. The concentration of ozone was such that 5 mg 18:1 was completely ozonized in 1½ min. After this treatment, ozonides were reduced by adding to the solution a few crystals of triphenylphosphine (2), and 15 μliter aliquots of the solution were analyzed in an F&M 402 gas chromatograph equipped with glass columns and flame ionization detectors. One 12 ft x ¼ in. column was packed with 5% LAC-2-R 446 (3) on Chromosorb W-AWDMCS and the other was 4 ft x ¼ in. packed with 5% Apiezon L on Chromosorb W-AWDMCS. Samples were injected onto the two columns simultaneously and the temperature was programmed from 80 to 200 C at 7.5 C/min.

When trienoic or tetraenoic esters were analyzed, the ozonolysis reaction was interrupted three to five times at 15 to 30 sec intervals. After each interval, the entire reaction mixture was reduced with triphenylphosphine and two 15 μliter aliquots were analyzed by GLC as above.

Equivalent chain lengths (ECLs) (3) were calculated with even chain length methyl esters (C_6-C_{22}) as standards using the equation:

$$ECL = S_1 + (S_2 - S_1)\ \frac{t_x - t_{s1}}{t_{s2} - t_{s1}}$$

where: S_1 = chain length of first standard ester; S_2 = chain length of second standard ester; $ts_{(1\ or\ 2)}$ = standard retention time: and tx = retention time of peak. The ECL of each peak was calculated using the two standards closest in retention time to that of the peak. ECLs and relative area percentages for each component were calculated by computer. In the case of monoenoic esters, the computer program provided identification of the fragments and the molar ratio of the parent esters.

In esters with (ω 3) bonds, the ozonides were formed at –23 C in CCl_4 and reduced with triphenylphosphine. The C_3 aldehyde formed was determined by GLC on a 9 ft x ⅛ in. stainless steel column packed with Porapak Q and held at 155 C in an F&M

[1]Presented at the AOCS Meeting, Chicago, October 1967.
[2]No. Utiliz. Res. Dev. Div., ARS, USDA.

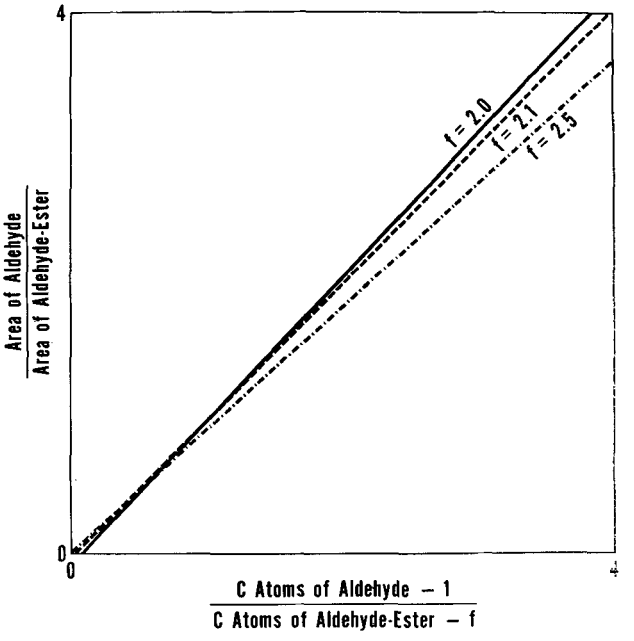

FIG. 1. Area vs. calculated response of monoenoic ester ozonolysis products from LAC-2-R 446 column. Variable f = nonresponding carbon atoms.

810 chromatograph equipped with a hydrogen flame detector (4).

Known fatty acid methyl esters of high purity were obtained from commercial suppliers, by preparation from known plant sources, or by synthesis.

RESULTS AND DISCUSSION

The products resulting from the ozonolysis reported by Stein and Nicolaides (2) are essentially all aldehydic in nature. Side products resulting from chain degradation or other anomalies (5) are almost completely absent from the mixture. This method has the further advantages that it involves no transfer of sample to a special reaction vessel, no catalyst preparation (6), no solvent purification, and employs standard injection techniques.

Our chromatographic method, which entails on-column injection into glass columns without preheaters, minimizes polymerization and loss of reactive ozonolysis products. Retention characteristics in the two columns, which have different stationary phases, provide identification of each fragment.

Relative Response of Flame Ionization Detectors to Ozonolysis Products

The aldehyde-ester fragments have been used in the past to quantitate the relative amounts of monoenoic esters (7,8); little has been done quantitatively to relate the aldehyde fragments to the aldehyde-ester fragments and to the parent ester. In the use of the flame ionization detector, quantitation is complicated by the unequal detector response to different organic molecules. The response has been reported to be proportional to the number of carbon atoms in the molecule. Dal Nogare and Juvet (9) describe a correction factor (C-factor) for conversion of area response to weight response. They equate this factor to (molecular weight)/(12 × number of carbon atoms) and suggest that carbon atoms bonded to oxygen be excluded from this calculation. A later report (10), however, indicates that methoxyl carbon atoms do respond to flame ionization detectors and that the absolute response of carboxyl carbon atoms is not clearly defined. In Figure 1 the ratios of the areas of aldehyde fragments to the areas of their corresponding aldehyde-esters from individual monoenoic esters are plotted against the ratios of the number of responding carbon atoms in each fragment. Aldehydic carbon atoms are assumed to have zero response while the number of nonresponding atoms in aldehyde-esters is set equal to the variable (f) so that a one to one relationship between the two ratios can be established. The line with f = 2.1 has unit slope. An additional assumption that the carboxyl carbon

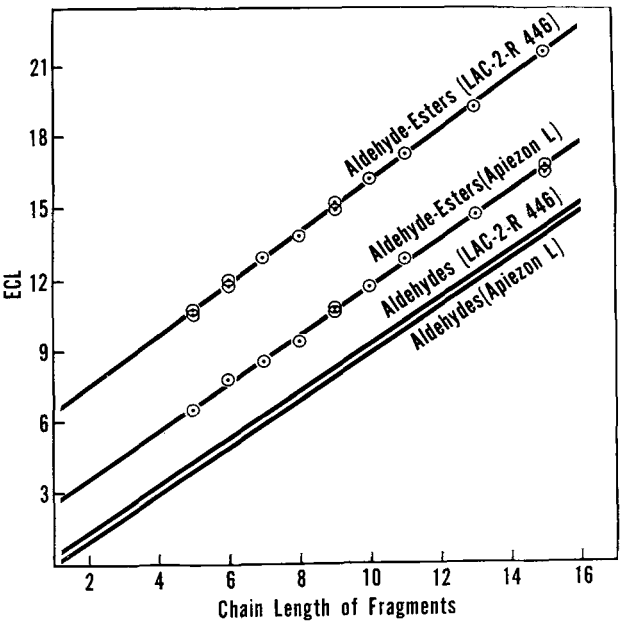

FIG. 2. Equivalent chain lengths (based on saturated methyl esters) of aldehydes and aldehyde-esters. Points on aldehyde lines were omitted for clarity but showed the same relation to the lines as in the aldehyde-ester plots.

is unresponsive and that the methoxyl carbon has nine-tenths the response of a methylene carbon would conform to this result. For practical use, it is adequate to consider that the carbonyl and carboxyl carbon atoms give no response and that the methoxyl carbon atoms respond equally with methylene carbon atoms. Therefore, the response of aldehyde fragments is proportional to number of carbon atoms in the molecule minus one and the aldehyde-ester response is proportional to its number of carbon atoms *minus two*.

Analysis of Monoenoic Esters

The products from reductive ozonolysis of monoenoic esters are aldehydes (A) and aldehyde-esters (AE). Ozonolysis of esters of known structure ($16{:}1^{3}$; $16{:}1^{9}$; 18:1 (individual isomers Δ 5 through Δ 12); $20{:}1^{5}$; $20{:}1^{11}$; $22{:}1^{13}$; $24{:}1^{15}$) produced a variety of fragments of known chain length as reference materials. ECLs (based on saturated methyl esters) of these fragments were determined from both Apiezon L and LAC-2-R 446 columns and were then plotted vs. their respective chain lengths. The straight line relationships evident in Figure 2 show that ECLs from the two columns can be used to identify ozonolysis fragments without further comparison with fragments from ozonized reference compounds. Methyl esters were used as standards instead of aldehydes because they are stable and readily available in high purity.

The ECLs of an aldehyde are essentially the same from both Apiezon L and LAC-2-R 446 columns, but those of aldehyde-esters differ markedly. Therefore, each component can be identified from the ECL data. Furthermore, components which overlap in one column are well separated by the other and quantitative relationships can be determined (11). The identification and quantitation of mixtures of monoenes of the same chain length and of simple mixtures of different chain lengths (if the relative proportions of esters of each chain length are known) are easily accomplished. In mixtures of monoenoic esters, peak areas from the ozonolysis fragments are used to calculate the mole percentages of the parent esters from the following relationships:

wt % = area % × C-factor, and C-factor = MW/ (responsive carbon atoms × 12).

Responsive carbon atoms = No. of carbon atoms — [carbonyl + carboxyl carbon(s)].

Therefore wt % $= \dfrac{\text{area \%} \times \text{MW}}{(N-f) \times 12}$, where N = total number of carbon atoms and f =

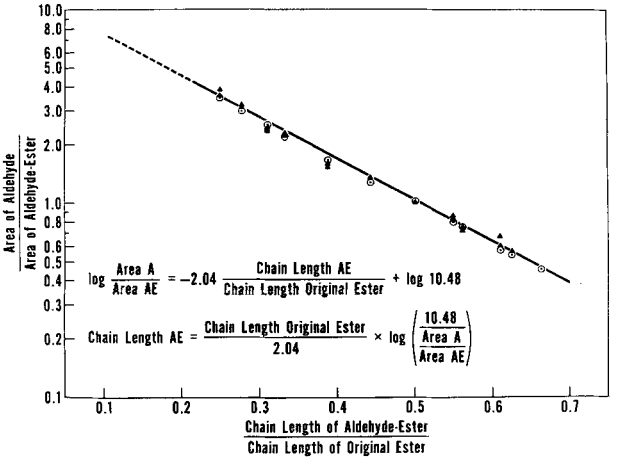

FIG. 3. Graphical determination of double bond position in monoenes from the ratio of peak areas of ozonolysis fragments: Δ from LAC-2-R 446 column, ⊙ calculated (see text).

number of carbonyl + carboxyl carbons.

$$\text{Mole Fraction, } X_a = \frac{\frac{\text{wt }\%_a}{MW_a}}{\sum_{x=1}^{x=n} \frac{\text{wt }\%_x}{MW_x}}$$

$$X_a = \frac{\frac{\text{area }\%_a \times MW_a}{MW_a \times (N-f)_a \times 12}}{\sum_{x=1}^{x=n} \frac{\text{area }\%_x \times MW_x}{MW_x \times (N-f)_x \times 12}}$$

$$X_a = \frac{\frac{\text{area }\%_a}{(N-f)_a}}{\sum_{x=1}^{x=n} \frac{\text{area }\%_x}{(N-f)_x}}$$

The mole fractions of the fragments produced on complete ozonolysis of a monoenoic ester are equal and, in a mixture of pure monoenes, their sum equals mole fraction of the parent ester in the original mixture. The ratios of the mole fractions of the fragments from the parent esters are the same as the ratio of the parent esters, even upon incomplete ozonolysis; the aldehyde fragments serve as well as the aldehyde-ester to establish the ratio.

A correlation was found when the logarithms of the ratios of the peak areas of the aldehydes to the corresponding aldehyde-esters were plotted vs. the ratios of the position of the double bond to the original chain length (Fig. 3). The calculated points, $\log[(N_A - 1)/(N_{AE} - 2)]$, vs. position of the double bond/chain length of original ester, are essentially the same as those found from the peak area ratios from the LAC-2-R 446 column. The relationship between the areas from fragments and the double bond position allows calculation of the position of the double bond from the peak areas of the aldehyde and aldehyde-ester if the parent chain length is known. Location of the double bond is a check on the identification of the aldehyde-ester made from retention data.

The ozonolysis procedure used permitted detection of some components not usually found from $KMnO_4/KIO_4$ oxidation (12). For example, analysis of $KMnO_4/KIO_4$ cleavage of 16:1[3] showed only one product, tridecanoic acid (13,14). Using the above ozonolysis procedure both 13A and 3AE were observed.

Analysis of Dienoic Esters

The products formed from the ozonolysis of dienoic methyl esters are aldehydes, aldehyde-esters and dialdehydes. To establish the positions of the olefinic bonds in pure single component esters, only correct identification of the aldehyde and aldehyde-ester fragments are necessary, although dialdehydes supply confirmatory evidence.

An example of a dienoic methyl ester from which the dialdehyde fragment is a major component is the 22:2[5,13] from *Limnanthes douglassii* (15, 16). The eight carbon dialdehyde (AA) has ECLs of 13.9 (LAC-2-R 446) and 8.8 (Apiezon L). A plot of ECLs of several

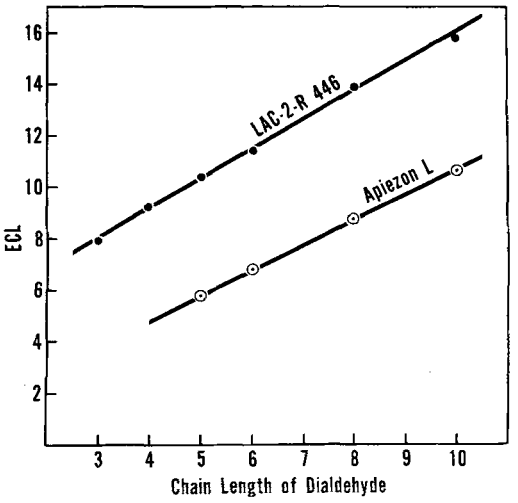

FIG. 4. Equivalent chain length (based on saturated methyl esters) of dialdehydes.

dialdehydes vs. their chain length is shown in Figure 4. The sources of these dialdehydes are listed in Table I. The detector response to dialdehydes is analogous to that of other aldehydic components in that neither carbonyl carbon atom responds to flame ionization. Therefore, 3AA has only one responding carbon atom and is not easily detected.

Another example of the superiority of the ozonolysis procedure for double bond location is shown by its application to the allenic diene, 5,6-octadecadienoic acid from *Leonotis nepetaefolia* (17). When the methyl ester of this acid was oxidized by $KMnO_4/KIO_4$ (12) only lauric acid was observed (17). Addition of acetic acid before oxidation was necessary before glutaric acid was detected (17). Ozonolysis revealed two products, 5AE and 12A, which clearly define the positions of the double bonds.

Analysis of Trienoic and Tetraenoic Esters

Complete ozonolysis of trienoic and tetraenoic esters and identification of the resulting aldehyde and aldehyde-ester fragments to reveal the structure of the esters is well documented (6,18). In both reports, the unsaturation in the esters is either all conjugated or all methylene-interrupted. Complete ozonolysis, therefore, resulted in aldehydes and aldehyde esters which served as identifying fragments.

TABLE I
Source of Known Dialdehydes

Dialdehyde	Source	Reference
3AA	$20:4^{5,11,14,17}$	25
4AA	$18:3^{5,9,12}$	21
5AA	Aldrich Chem. Co.	—
6AA	$20:3^{5,11,14}$	25
8AA	$22:2^{5,13}$	17
10AA	$18:3^{5,8,16}$	22

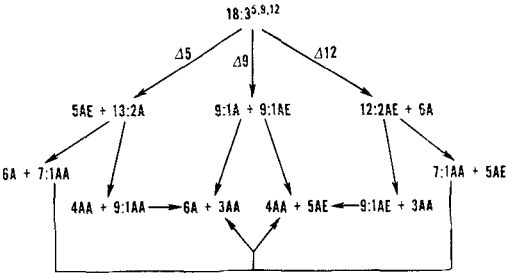

FIG. 5. Reaction scheme for ozonolysis of $18:3^{5,9,12}$.

If an ester has isolated olefinic bonds, complete ozonolysis would not unequivocally locate the positions of unsaturation. For example, $20:4^{5,11,14,17}$ ester, upon complete ozonolysis, yields 5AE, 3A, 6AA, and 3AA as the only products. These products could also result from complete ozonolysis of two other esters: $20:4^{5,8,11,17}$ and $20:4^{5,8,14,17}$. Schlenk solved this problem in the characterization of the $20:4^{5,11,14,17}$ ester from *Ginkgo* lipids (19) by ozonolysis of the ester before and after alkali-isomerization, thereby locating the bonds that were methylene-interrupted. By interrupting the ozonization at various stages before completion, unsaturated fragments (20) are produced as well as the products from complete reaction. The unsaturated intermediates serve to locate the interior olefinic bonds. For example, the "interrupted ozonolysis" method, when applied to the $20:4^{5,11,14,17}$ ester, produced two structure-determining unsaturated fragments, 11:1AE and 6:1A. These fragments together with the end products (5AE, 3A, 6AA, and 3AA) determine the locations of all double bonds.

A simpler ester, $18:3^{5,9,12}$ (21), serves to illustrate the many possible unsaturated fragments that can form during interrupted ozonolysis (Fig. 5). The fragments necessary to locate the double bonds correctly are: 5AE, 6A, 9:1AE, or 9:1A (Fig. 6 and 7). The other fragments supply confirmatory evidence. The 3AA from an ester such as the $20:4^{5,11,14,17}$ or $18:3^{9,12,15}$ is quite evident as two of these fragments are produced per ester molecule.

Identification of unsaturated fragments was made on the basis of their ECLs on both the LAC-2-R 446 and the Apiezon L columns. The ECLs of fatty acid methyl esters are

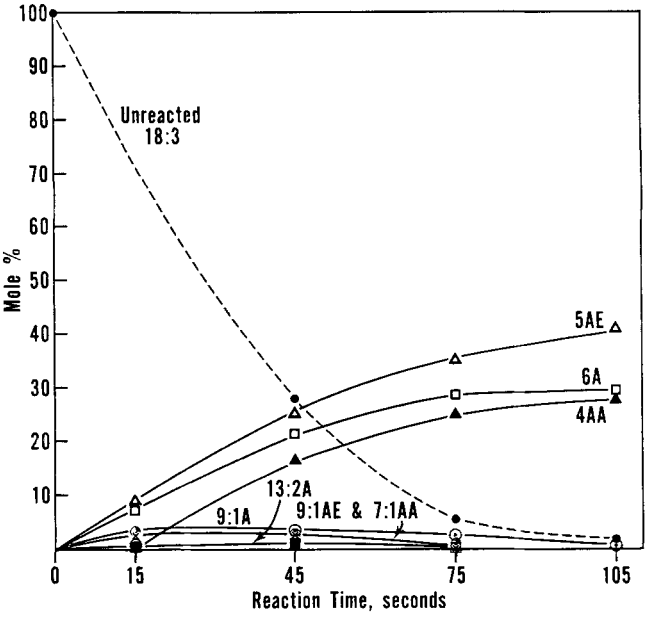

FIG. 6. Concentration of ozonolysis fragments from $18:3^{5,9,12}$ vs. reaction time.

greater by 0.4 units per double bond on the polar LAC-2-R 446 column than their saturated analogues and unsaturated esters usually have ECLs approximately 0.3 units less than their saturated analogues on the Apiezon L column (3). Similarly, 9AE has an ECL of 15.0 on LAC-2-R 446 while the ECL of the 9:1AE is 15.4; the ECLs on Apiezon L are: 9AE = 10.8 and 9:1AE = 10.5. This relationship holds for all chain lengths. Table II shows the ECLs of the components formed from ozonolysis of $18:3^{5,9,12}$ ester.

Confirmation of the unsaturated nature of the components formed early in the ozonization is provided by their decrease and disappearance as ozonolysis continues. Figure 6 illustrates this phenomenon. As expected, the molar amounts of the 5AE, 4AA, and 6A increase throughout the experiment, but the 9:1A, 9:1AE, 7:1AA, and 13:2A increase for a time and then de-

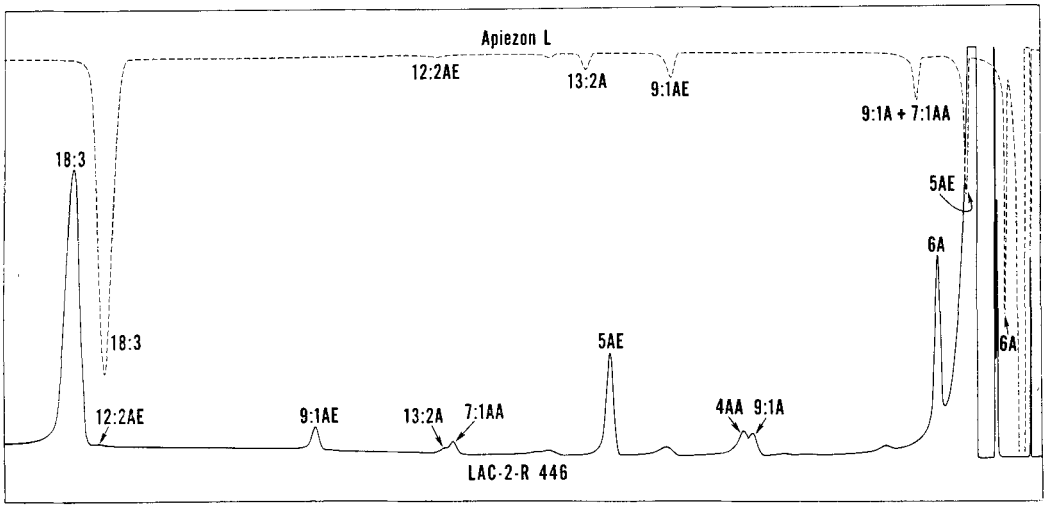

FIG. 7. GLC of ozonolysis products of $18:3^{5,9,12}$ after 45 sec reaction time from LAC-2-R 446 and Apiezon L columns.

TABLE II

Ozonolysis Products of $18:3^{5,9,12}$

Reaction time	Component	ECL LAC-2-R 446	ECL Apiezon L	Area % (LAC-2-R 446)	Ionizable carbons	Mole %
sec						
45	6A	5.2	5.2	12.0	5	20.3
	5AE	10.9	6.8	11.8	4	25.0
	9:1A	8.7	7.8	3.1	8	3.3
	9:1AE	15.4	10.5	2.7	8	2.9
	13:2A	13.3	11.8	1.1	12	0.8
	12:2AE	18.3	13.3	tr	12	tr
	7:1AA	13.2	7.6	1.8	5	3.0
	4AA	9.1	4.0	4.0	2	16.9
	18:3	19.4	17.7	59.0	18	27.8
105	6A	5.2	5.2	37.3	5	29.6
	5AE	10.9	6.8	41.3	4	41.0
	9:1A	8.7	7.8	0.2	8	0.1
	4AA	9.1	4.0	14.0	2	27.7
	18:3	19.4	17.7	7.1	18	1.6

crease. The 9:1A is the unsaturated fragment produced in the largest amount and is the only one remaining after 105 sec (Table II).

The allenic triene, $18:3^{5,6,16}$ from *Lamium purpureum* (22), was investigated by the interrupted ozonolysis method. After 30 sec ozonization, four major components were detected: 5AE, 10AA, 16:2AE (ECL: LAC-2-R 446 = 23.8, Apiezon L = 17.4), and unreacted 18:3. The 16:2AE was 28% of the total peak area. The trienoic ester disappeared completely before a significant decrease (to 19% at 2 min) in the 16:2AE peak area was observed. Three minutes were required to completely ozonize the 16:2AE. Since the double bonds of this component are allenic, it appears that such bonds react more slowly than isolated bonds.

Three other esters with isolated double bonds were analyzed. These and their identifying products are as follows: $18:3^{3,9,12}$ (23), 3AE, 6A, 6AA, 9:1A and 9:1AE; $20:3^{5,11,14}$ (24,-25), 5AE, 6AA, 6A, 9:1A and 11:1AE; and $20:4^{5,11,14,17}$ (19,24,25), 5AE, 3A, 6AA, 6:1A and 11:1AE.

ACKNOWLEDGMENTS

C. R. Scholfield supplied several authentic monoenoic esters. Methyl 5,13-docosodienoate was obtained from F. G. Dollear, Southern Regional Research Laboratory, New Orleans, La. We thank T. K. Miwa for his helpful discussions.

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[Received July 2, 1968]

Deposition of Dietary Epoxides in Tissues of Rats[1]

J. A. FIORITI, N. BUIDE and R. J. SIMS, Technical Center, General Foods Corporation, White Plains, New York 10602

ABSTRACT

Epididymal fat pad lipids from rats fed trivernolin at the 4.8% level in the diet for 90 days were found to contain 6.1% epoxyoleic acid. No epoxides were detected in the serum lipids and only trace amounts were found in the liver lipids. TLC, GLC and a specific color reaction with picric acid were used to identify this fatty epoxide in the tissues. Epoxyoleic acid was shown to be present as a mixture of predominately monovernoloyl triglycerides with some divernoloyl triglycerides and small amounts of trivernolin. Lipase hydrolysis of the first two triglycerides has demonstrated that the vernoloyl groups are present mostly in the 1,3 positions. In a separate experiment, rats were fed cholesterol epoxide at the 0.5% and 1.5% levels in the diet for 90 days. TLC and GLC examination of lipids from these rats failed to reveal the presence of any cholesterol epoxide. Only one-half of the sterol fed could be accounted for in the fecal lipids.

INTRODUCTION

The formation of epoxides during the heating of vegetable oils in air has been demonstrated by prior work (1, 2). Likewise, autoxidation of cholsterol has been shown to give rise to cholesterol epoxide (5α-cholestane-5α, 6α-epoxy-3 βol) (3). Although some feeding studies have been carried out with these and similar epoxides (4, 5), the biological activity and the metabolic fate of such epoxides are largely unresolved. Holman and co-workers have shown that epoxyoleic (vernolic) acid does not function as an essential fatty acid, (6) and that it is absorbed from the digestive tract and deposited in fatty tissues (7).

In the study reported here, a 90 day feeding experiment was carried out to determine the level of epoxides which rats would tolerate in their diet. This paper reports the analysis of lipids resulting from the feeding study. The concomitant physiological effects on the rat will be discussed in a following report (8).

EXPERIMENTAL PROCEDURES

A trivernolin concentrate was prepared from *Vernonia anthelmintica* seed oil by overnight crystallization at -10 C from four volumes of petroleum ether (bp 30-60 C). After the supernatant liquid had been decanted, the crystals were melted and freed from solvent on a steam bath in a nitrogen stream, under vacuum. The oil was processed in 1-2 kg batches which were then combined to give the product described in Table I. The recovery averaged 56% of a concentrate which, based upon an analysis for oxirane oxygen, contained 83.9% trivernolin.

Cholesterol epoxide was prepared by oxidizing cholesterol (American Cholesterol Co., Inc., Edison, NJ) with 80% *m*-chloroperbenzoic acid as described in a recent publication (9). The procedure was modified slightly so as to combine the reduction and removal of excess reagent by washing the reaction mixture with 5% NaOH. Several batches of cholesterol (300-750 g) were oxidized and then combined to give the product described in Table I. The yield of epoxide ranged from 50-60%.

Three groups of 20 Sprague-Dawley rats were fed a commercially prepared diet (Wayne Chow, Carworth Farms, New City, NY). In two of these groups the diet was supplemented with 1.6% and 4.8% of trivernolin. After 90 days the animals were killed and the sera, livers and epididymal fat pads from each group were pooled. The lipids were extracted with chloroform-methanol (2:1) and analyzed for epoxides. Fecal samples were collected, pooled, extracted and the extracts analyzed for epoxides.

In a separate experiment two groups of 20 animals were fed cholesterol epoxide at the 0.5% and 1.5% levels in the diet. After 90 days the animals were killed and the tissues from each group were pooled. In this case the sera, livers, epididymal fat pads, spleen, kidney and fecal lipids were analyzed.

Details of the analytical methods used, including transmethylation, TLC and GLC, are given elsewhere (10). Identification of epoxide-containing spots on a TLC plate by visualization with picric acid is described in a previous publication (11).

[1]Presented in part at the AOCS Meeting, Chicago, October 1967.

TABLE I
Characteristics of Epoxides Fed to Rats

Trivernolin concentrate	
Oxirane oxygen	4.34%[a]
Trivernolin content	83.9%
Peroxide value	11.5 meq/kg[b]
Free fatty acids	2.0%[c]
Unsaponifiables	2.5%[d]
Cholesterol epoxide	
Oxirane oxygen	3.72%[a]
Sterol epoxide content	93.7%
Impurities	ca. 5.0% cholesterol
Melting point	139-142C
Peroxide value	2.0 meq/kg[b]
Free acid content	0.03%[c]

[a] AOCS Tentative Method Cd 9-57.

[b] AOCS Official Method Cd 8-53.

[c] Expressed as vernolic acid or *m*-chloroperbenzoic acid, respectively.

[d] AOCS Official Method Cd 6a-40.

RESULTS AND DISCUSSION

Trivernolin Feeding

Epoxides were readily identified in the rat lipids after trivernolin feeding. The TLC plate in Fig. 1 shows three distinct epoxide-containing spots in the epididymal fat pad lipids from the rats fed trivernolin at the 4.8% level (lane 6c, d and e). These spots were visualized by spraying the plate with picric acid and were singled out on the plate by circling the reddish-orange areas with a pencil.

By GLC (10) the lipids spotted on lane 6 contained 6.1% vernolic acid. One epoxide-containing spot, e, is also visible in lane 5 which contains the epididymal fat pad lipids from rats fed 1.6% trivernolin. The epididymal fat pad lipids taken from the control group (lane 4) show no epoxide-positive spots. Lane 7 contains liver lipids from the high level trivernolin feeding. No epoxide-containing spots are visible in this lane. However, when these liver lipids were streaked at the origin on a TLC plate, developed and the epoxide region scraped from the plate, a concentrate was obtained which on respotting and developing did show a small amount of epoxide-positive material at e, the monovernoloyl triglyceride region. Lane 8 contains fecal lipids from rats which had been on the diet high in trivernolin. As expected, it shows sterols (between b and c) and trivernolin (c). Also included on this plate is the original trivernolin concentrate (lane 1) which contains a small amount of a second epoxide spot at d, divernoloyl triglycerides. Lane 2 shows vernolic acid, which in the nonacidic solvent system

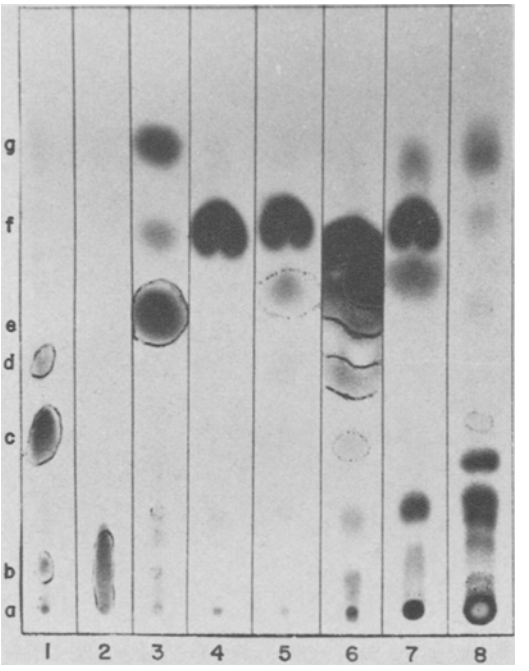

FIG. 1. TLC of rat lipids from trivernolin feeding. Lane 1. Trivernolin (TV) concentrate; 2. Vernolic acid; 3. Transmethylated TV concentrate; 4. Epid. fat pad lipids, control group; 5. Epid. fat pad lipids, 1.6% TV group; 6. Epid. fat pad lipids, 4.8% TV group; 7. Liver lipids, 4.8% TV group; and 8. Fecal lipids, 4.8% TV group. a. Monoglycerides; b. Vernolic acid; c. Trivernolin (TV); d. Divernoloyl triglycerides; e. Methyl vernolate or monovernoloyl triglycerides; f. Common triglycerides; and g. Unsaponifiables.

used here moves as a streak to a short distance from the origin. An additional material included on the plate is transmethylated trivernolin (lane 3) to which squalane (g) had been added as an internal standard prior to GLC assay. Epoxides could not be detected in the serum lipids from animals fed 4.8% trivernolin even when the material was streaked on a TLC plate, the plate developed and the epoxide region removed, extracted and rechromatographed.

The epididymal fat pad lipids from the 4.8% trivernolin feeding were then separated into four fractions by column chromatography and preparative TLC on silica gel (10). In addition to the common triglycerides, these lipids were found to contain mono-, di- and trivernoloyl triglycerides. A quantitative assay of these epididymal fat pad lipids and of the four fractions from them was carried out, after

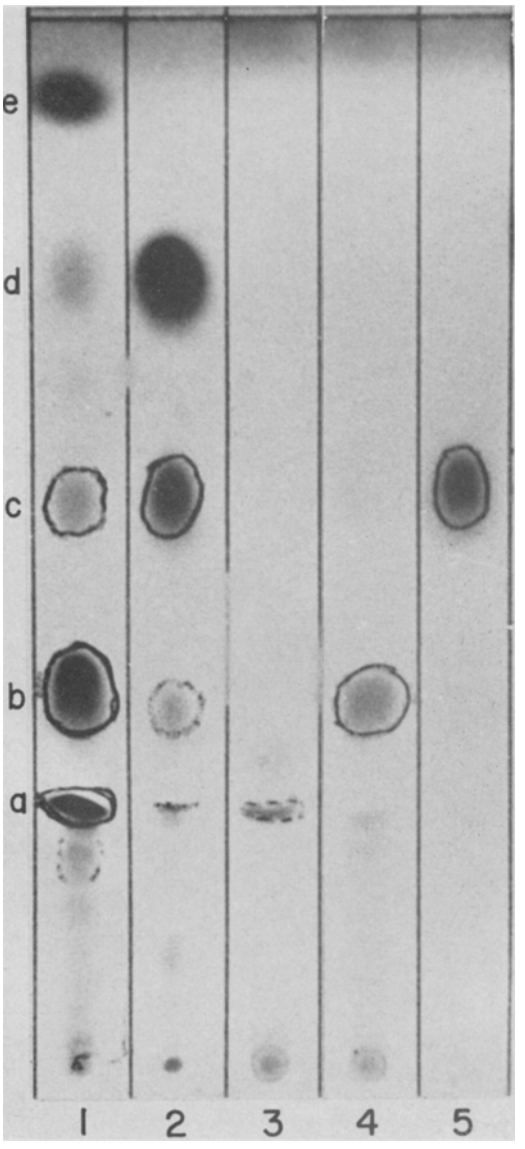

FIG. 2. TLC of fractionated epididymal fat pad lipids—the circled spots are the epoxide-containing moieties. A dotted circle indicates trace amounts. Lane 1. *Vernonia* seed oil; 2. Epididymal fat pad lipids, 4.8% TV feeding; 3. Trivernolin from 2; 4. Divernoloyl triglycerides from 2, and 5. Monovernoloyl triglycerides from 2. a. Trivernolin; b. Divernoloyl triglycerides; c. Monovernoloyl triglycerides; d. Common triglycerides; and e. Unsaponifiables.

transmethylation, by GLC. The results of this analysis on the total fat and on the three epoxide-containing fractions, are given in Table II.

The trivernolin fraction represents less than 1% of the total lipid and, from the data, is

TABLE II

GLC Analysis of Epididymal Fat Pad Lipids From Rats Fed Trivernolin Concentrate[a]

	% Composition[b]			
Fatty acids	Total fat	Trivernolin[c]	Divernoloyl triglycerides	Monovernoloyl triglycerides
C_{14}	2.5	8.6	1.2	1.8
C_{15}?	0.4	0.2	0.2	0.2
C_{16} & $C_{16:1}$	34.9	32.7	15.6	25.1
C_{18} & $C_{18:1}$	27.1	21.6	11.8	20.4
$C_{18:2}$	27.4	6.8	6.3	24.4
$C_{18:3}$	0.8	trace	nil	0.5
$C_{20:4}$?	0.3	1.0	nil	0.7
Vernolic	6.1	29.0	64.8	31.7

[a] Trivernolin fed at 4.8% level.

[b] GLC analysis performed on Carbowax 20M (10). The results given represent the average of two determinations.

[c] This impure fraction represents <1% of the total lipid.

obviously impure. The TLC separation of these epididymal fat pad lipids is shown in Fig. 2. Included on this plate is *Vernonia* seed oil (lane 1), the epididymal fat pad lipids (lane 2), and the three epoxy-containing fractions obtained by column chromatography (lanes 3-5). By visual estimation as much as 90% of the total vernolic acid in these lipids was present as monovernoloyl triglycerides (lane 5, c).

To determine the distribution of the vernolic acid in the epoxide-containing fractions described above, lipase hydrolysis experiments were carried out. Lipase digestions of trivernolin (13), and of mono- and divernoloyl triglycerides (12,14) indicate that the enzyme attacks these epoxy triglycerides in the conventional manner, by preferential, but not exclusive, splitting at the 1,3 positions. Our previous experience with *Vernonia* oil (10) has demonstrated that most of the divernoloyl triglycerides are in the VVC form, while most of the monovernoloyl triglycerides are in the VCC form (V represents vernolic and C a common fatty acid). This conclusion is based upon the reasoning that the symmetrical triglyceride CVC should yield only one diglyceride, whereas two were actually obtained by lipolysis.

The present work has shown that the VCC distribution also holds for the monovernoloyl triglycerides from epididymal fat pad lipids in the trivernolin feeding experiment. TLC indicates that the monovernoloyl triglyceride fraction, on lipolysis, gives both common diglycerides and those containing vernolic acid. This conclusion is strengthened by GLC analyses of the lipase hydrolysate which also reveals a CC and a VC diglyceride. These results are reflect-

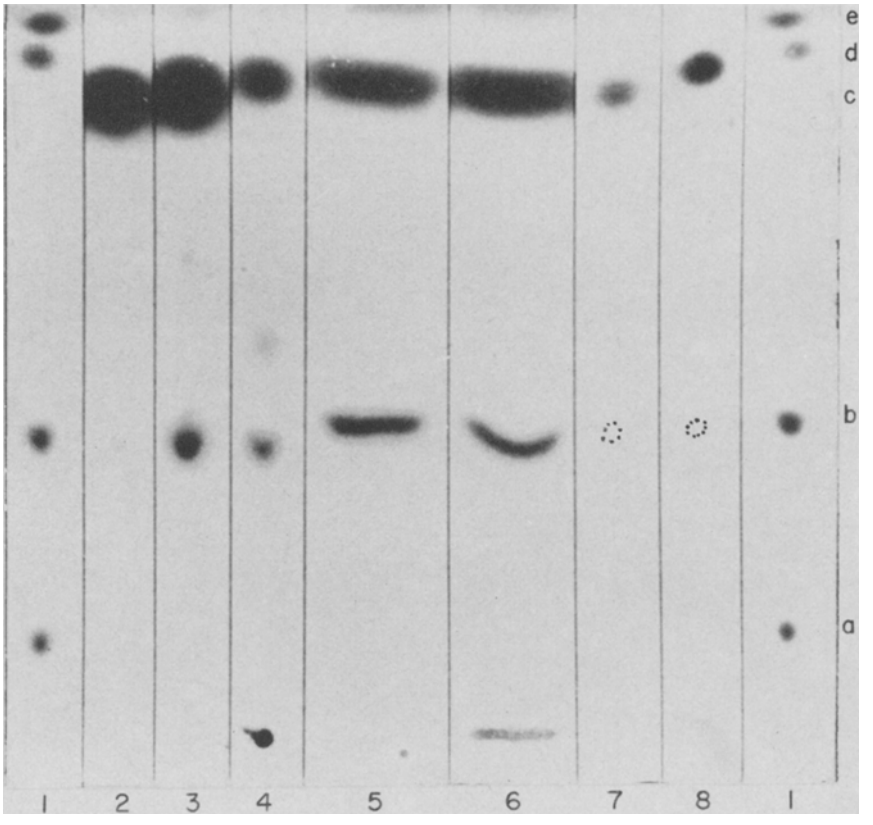

FIG. 3. TLC of lipids from rats fed cholesterol epoxide. Lane 1. Mixture of a, b, d and e below; 2. Epididymal fat pad lipids; 3. Liver lipids; 4. Kidney lipids; 5. Serum lipids; 6. Spleen lipids; 7. Transmethylated cottonseed oil; and 8. Cottonseed oil. a. Cholesterol epoxide; b. Cholesterol; c. Triglycerides; d. Epoxycholesteryl palmitate; and e. Cholesteryl palmitate.

ed in Table III in which the monoglycerides obtained by lipase digestion of epididymal fat pad lipids are compared with those obtained from *Vernonia* oil.

TABLE III

Distribution of Vernoloyl Groups in Epididymal Fat Pad Lipids

Sample	Monovernolin, mole % in monoglycerides	Proportion of vernoloyl groups in 2-position[a]
Trivernolin	100	33
Divernoloyl triglycerides from *Vernonia* oil	72	36
EFPL[b]	28	14
Monovernoloyl triglycerides from *Vernonia* oil	8	8
EFPL[b]	20	20

[a] Proportion in 2 position $= \frac{\text{mole \% in monoglycerides} \times 100}{3 \times \text{mole \% in sample}}$ (12).

[b] Epididymal fat pad lipids from 4.8% trivernolin feeding.

The predominant epoxyglyceride fraction from rat lipids, the monovernoloyl triglycerides, was found to yield, on lipolysis, a monoglyceride containing 80% of common fatty acids and only 20% of vernoloyl groups.

From the data assembled here it is evident that trivernolin fed to rats is deposited predominately as monovernoloyl triglycerides in which the epoxyoleic acid is present mostly in the 1 position. The major pathway for triglyceride synthesis in the animal organism is known to involve direct acylation of 2-monoglycerides (15). Thus the marked preference of the vernolic acid for the 1 position in epididymal fat pad lipids argues for a possibly abnormal metabolic pathway with epoxyglycerides.

Cholesterol Epoxide Feeding

The epididymal fat pad lipids, the liver, kidney, serum and spleen lipids from the rats fed cholesterol epoxide at the 1.5% level were

chromatographed on a TLC plate along with some known sterols as shown in Fig. 3. This figure indicates the presence of cholesterol (b) in liver, kidney, serum and spleen lipids but shows an absence of cholesterol epoxide (a). As an indication of the sensitivity of the TLC method, lanes 7 and 8 show the traces of cholesterol present in cottonseed oil and in methyl esters from cottonseed oil (dotted circles) (17). Since it was possible that cholesterol epoxide might have appeared as a fatty acid ester, both epoxycholesteryl palmitate (d) and cholesteryl palmitate (e) were included in the mixture of sterols spotted in lane 1. The Rf of triglycerides (c) is quite close to that of epoxycholesteryl palmitate (d) which makes a differentiation between them on a TLC plate almost impossible. Nor can a distinction be made between them by recourse to the usual sterol color reactions (3). Unsaturated triglycerides were also found to give a brown color, similar to epoxycholesteryl palmitate, with H_2SO_4, $SbCl_3$, anisaldehyde and other sterol reagents (16). For this reason each of the lipid samples was transmethylated, streaked on a TLC plate and developed. The band having an Rf equal to cholesterol epoxide was scraped from the plate, eluted and rechromatographed. Conversion to the free sterol and concentration by the above technique failed to reveal even a trace of cholesterol epoxide in any of these lipids. By a GLC analysis of the fecal lipids collected from these rats approximately 50% of the ingested cholesterol epoxide could be accounted for. The fate of the remaining sterol is currently being investigated.

ACKNOWLEDGMENTS

Supported by contract No. PH 43-63-1165, National Institute of Health. I. A. Wolff of the Northern Regional Research and Development Division, U.S. Department of Agriculture provided the *Vernonia* seed oil. B. Zeitlin and C. Mauge furnished the rat lipids.

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[Received February 15, 1968]

Enterohepatic Circulation of Cholic Acid and Cholesterol Metabolism in the Rat[1]

LUDVIK PERIC-GOLIA, Department of Pathology, University of Utah College of Medicine and Veterans Administration Hospital, Salt Lake City, Utah 84113

ABSTRACT

A role for cholic acid in the regulation of body cholesterol levels is emphasized by the following experiment and findings. Fifteen rats with biliary and duodenal fistulae were divided into three groups. 4-^{14}C-cholesterol was admistered intravenously to all animals on day 1. Two groups received a duodenal infusion of either sodium taurocholate or of cholesterol at the same time and thereafter daily. Bile samples were collected daily for 15 consecutive days. Cholic, chenodeoxycholic acid and cholesterol were the major labeled compounds in the bile. Labeled cholic acid disappeared from the bile of control animals after day 8 while it persisted in the group receiving unlabeled cholic acid up to day 15. The decrease of specific radioactivity of labeled biliary cholic acid in the rats receiving unlabeled cholesterol corresponded to that of control animals. A significant increase in the concentration of cholesterol was found in the plasma of animals receiving cholic acid and in the liver of those receiving unlabeled cholesterol.

INTRODUCTION

The processes leading to the formation of cholesterol are well explored (1) but less is known about the mechanisms regulating the rate of its synthesis and degradation. Working with an in vitro system, Siperstein and Fagan (2) demonstrated inhibition of β-hydroxy-β-methylglutaryl reductase in the liver of rats kept for a short time on a diet supplemented with small amounts of cholesterol, thus confirming the earlier observations of Gould and Popják (3), Bucher et al. (4) and Siperstein and Guest (5). Identical mechanisms have been observed in the liver of other mammals including man (6), but an inhibition of the regularly occurring extrahepatic mevalonate synthesis (7) by dietary cholesterol has not been demonstrated in any species. It has been proposed that cholesterol can inhibit the reduction of β-hydroxy-β-methylglutarate in the liver only after cholesterol passes through the intestinal wall and forms a specific cholesterol lipo-protein complex since this inhibition cannot be shown after parenteral administration of cholesterol or if body cholesterol is elevated following increased endogenous production of the sterol (6).

Due to the aforementioned limitations of the control of cholesterol biosynthesis at the step of the conversion of β-hydroxy-β-methylglutarate to mevalonate, it does not seem likely that this control is the sole mechanism regulating concentration and distribution of cholesterol in plasma and tissues. Cholesterol content of plasma and tissues appears to depend upon the balance between the intake (exogenous) and biosynthesis on one side, and degradation and excretion on the other side. Only a small portion of cholesterol is excreted unchanged and a negligible amount is transformed into steroid hormones. Bile acids are the principal end products of cholesterol metabolism (8). The loss of these acids via the intestinal tract will increase their formation from cholesterol and as a consequence plasma and tissue cholesterol will decrease (9). A relation between the rate of formation of bile acids and cholesterol levels in plasma and tissues exists in several mammalian species (10) and studies in vitro indicate a selective effect of different bile acids on cholesterol metabolism (11). Experimental data reported by Dietschy (12) suggest that bile salts affect cholesterogenesis in the small bowel by, presumably, inhibiting the formation of mevalonic acid. It has been found that following intravenous injection of 4-^{14}C-cholesterol to guinea pigs with biliary fistulae, labeled cholic (3α,7α,12α-trihydroxycholanic) acid appears slowly in the bile and undergoes a prolonged enterohepatic circulation compared with the relatively rapid excretion of labeled chenodeoxycholic (3α,7α-dihydroxycholanic) and 7-ketolithocholic (3α-hydroxy,7-ketocholanic) acids observed in such animals (13). The half-life of labeled cholic acid is prolonged and its formation from cholesterol reduced in patients with hypercholesterolemia (14). No elevation of plasma cholic acid can, however, be measured in this patient category (15).

The present study concerns the relation be-

[1] A preliminary report was presented at the 49th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, 1965.

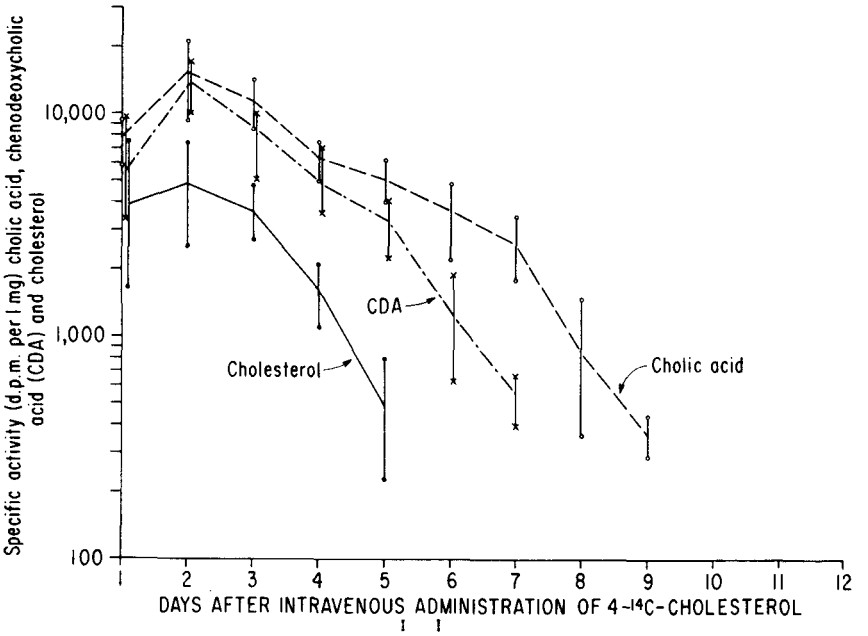

Fig. 1. Mean specific radioactivity (d.p.m./mg) of cholesterol, cholic acid and chenodeoxycholic acid (CDA) isolated from bile, in control animals given 4-^{14}C-cholesterol intravenously on day 1. ● ●, ○ ○, x x, range of values.

tween concentration of cholic acid in enterohepatic circulation and the rate of conversion of cholesterol into bile acids in the rat.

EXPERIMENTAL PROCEDURES

Male Sprague-Dawley rats weighing 300-400 g were used. Animals were fed Purina rat chow and water ad lib. Biliary fistulae were performed in a manner similar to that previously described in guinea pigs (13), but the surgical procedure was modified because of the smaller caliber of the bile duct and the absence of the gallbladder in the rat. One polyethylene catheter (PE-10) was inserted into the proximal and another into the distal portion of the common bile duct which was then ligated and cut between the two sites of the insertion of the catheters. The free ends of the catheters were passed under the skin to the back of the animal, pulled through a cutaneous opening and connected to allow enterohepatic circulation. Animals showing loss of weight, bacterial growth in cultures of bile, obstruction of catheters by biliary precipitates or any gross or microscopic evidence of disease at the post mortem examinations were excluded from this study.

Fifteen healthy animals were divided into three equal groups. Each of the 15 animals received 0.1 μc (6.6 μg) 4-^{14}C-cholesterol, dissolved in 0.2 ml 40% ethanolic saline per 100 g body weight into the tail vein 48 hr after the completion of the surgical procedure. The animals of two groups received at the same time and thereafter daily a duodenal infusion of 2 mg sodium taurocholate or 2 mg cholesterol respectively, dissolved or suspended in 2 ml 12.5% ethanolic saline per 100 g body weight. Suspensions of cholesterol were very fine and remained stable for several hours. The five control rats received only 12.5% ethanolic saline by duodenal infusion. All infusions were performed with a manually operated syringe over a 10 min period. Continuous enterohepatic circulation was maintained through the experiment except for short daily interruptions between 8 and 9 a.m. During this time bile samples of about 0.5 ml each were collected. Thus the loss of bile throughout the experiment was minimal. The animals were killed with a neck clamp 15 days following the injection of labeled cholesterol. Blood was withdrawn by cardiac puncture, and liver, kidneys and aorta were collected. Bile, plasma and tissues were frozen immediately and kept frozen until analyzed.

Bile acids were determined in the bile essentially as previously described (13,16). All samples were diluted to 50 ml with 1N sodium hydroxide, boiled 5 hr, acidified to pH 1 with conc. hydrochloric acid and extracted 3 times with 5 volumes ethyl ether each time. The pooled extracts were washed with water until

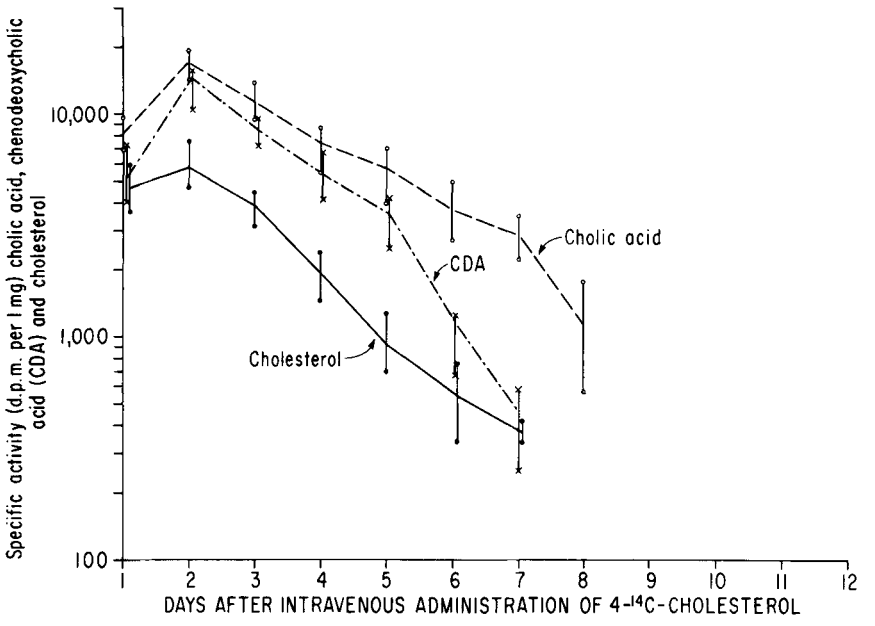

Fig. 2. Mean specific radioactivity (d.p.m./mg) of cholesterol, cholic acid and chenodeoxycholic acid (CDA) isolated from bile, in animals receiving unlabeled cholesterol by duodenal infusion. The symbols are the same as in Figure 1.

free of hydrochloric acid and the ethyl ether evaporated to dryness. The content of free bile acids in these samples was evaluated by paper chromatography (17). Chromatographic mobility of individual bile acids in the extract was compared to that of standard compounds, chromatographed on duplicate strips and sprayed with 10% phosphomolybdic acid in 70% ethanol following chromatography. The paper strips used for biological samples were scanned for radioactivity. The radioactive zones of these strips corresponding to phosphomolybdic acid positive spots on the standard strips were eluted with chloroform-methanol (1:1) and the eluate evaporated to dryness. The residue was treated with 65% sulfuric acid and the resulting chromogen measured by spectrophotometry (18). The cholesterol content of bile, plasma and homogenized tissues was determined spectrophotometrically following previously described extraction and purification procedures (19).

In order to determine purity of the radioactive products, the isolated bile acids and cholesterol were mixed with authentic compounds and crystallized several times: cholic acid from aqueous methanol, chenodeoxycholic acid from mixture of ethyl acetate and petroleum ether, cholesterol from acetone and from aqueous methanol. Specific activity (d.p.m./mg) remained constant through repeated crystallizations. Radioactivity of the samples was measured in a scintillation counter.

RESULTS

Isolated ^{14}C-cholic and ^{14}C-chenodeoxycholic acid contained 80-95% of the total radioactivity present in the bile while 10-20% of the radioactivity in the biliary samples chromatographed like cholesterol. No other labeled material was present in sufficient amount to be identified. Biliary excretion of all labeled compounds determined (cholic, chenodeoxycholic acid, cholesterol) was most abundant in all animal groups on the second day after the intravenous admistration of 4-^{14}C-cholesterol. The data are presented in Figures 1-3 as specific activity (d.p.m./mg) of cholic, chenodeoxycholic acid and cholesterol isolated from bile.

Specific radioactivity of cholic and chenodeoxycholic acid in control animals (Fig. 1) decreased rapidly following the admistration of 4-^{14}C-cholesterol; labeled cholic acid disappeared from the bile after the ninth day and labeled chenodeoxycholic acid after the seventh day. Specific radioactivity of biliary cholesterol in control animals was considerably lower than that of the two bile acids. The last samples containing labeled cholesterol were collected on the fifth day after the injection of 4-^{14}C-cholesterol.

Specific radioactivity of cholic acid, chenodeoxycholic acid and cholesterol in the group receiving duodenal infusion of unlabeled cholesterol (Fig. 2) did not differ significantly from

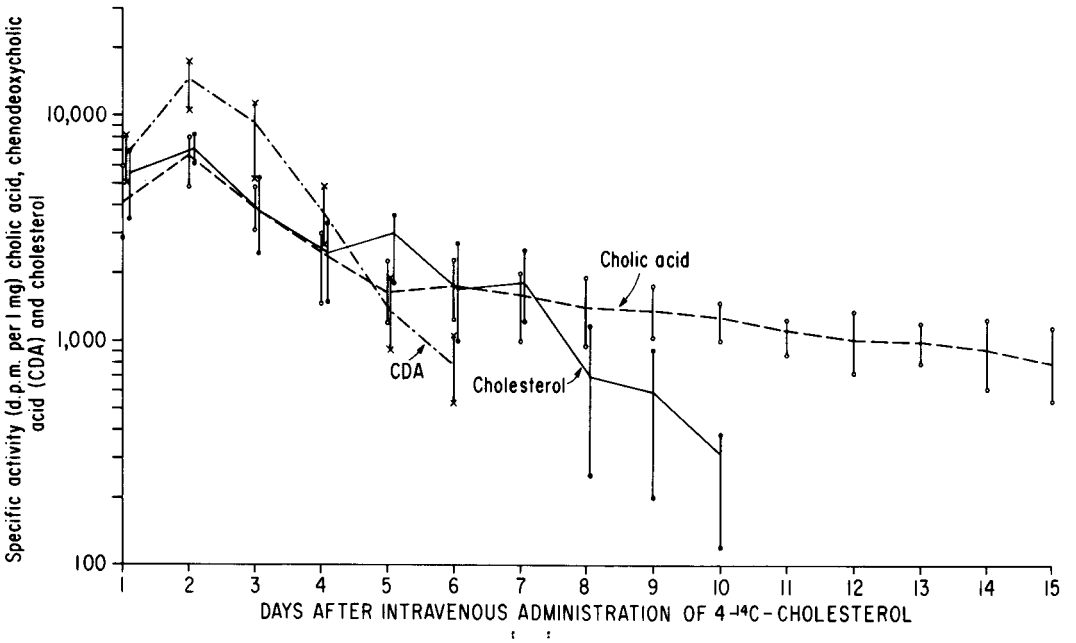

Fig. 3. Mean specific radioactivity (d.p.m./mg) of cholesterol, cholic acid and chenodeoxycholic acid (CDA) isolated from bile, in animals receiving unlabeled cholic acid by duodenal infusion. The symbols are the same as in Figure 1.

that observed in the control group.

In the animals receiving non-labeled cholic acid by duodenal infusion (Fig. 3) the daily excretion of labeled cholic acid was never as high as in the control group. Its specific radioactivity decreased rather rapidly until the fifth day of the experiment, thereafter the decrease per day was very moderate. Labeled cholic acid persisted in the bile of this group until the 15th day following the administration of labeled cholesterol. This was the only group where labeled cholic acid could be measured nine days after the administration of 4-^{14}C-cholesterol. The decrease of specific radioactivity of chenodeoxycholic acid in this group was practically identical to that in the control group. The specific radioactivity of cholesterol in the bile of animals with supplement of cholic acid was initially higher than in the other two groups but samples obtained after the 10th day contained only small and quite variable amounts of labeled cholesterol.

Cholesterol concentrations in plasma, liver, kidneys and aorta are listed in Table I. A significant increase was noted in the plasma of the group receiving cholic acid and in the liver of the group receiving unlabeled cholesterol.

DISCUSSION

The rate of formation of bile acids from cholesterol is inversely proportion to their concentration in the enterohepatic system (20). The present study demonstrates a delay of conversion of exogenous cholesterol to cholic acid, a prolonged biliary excretion of the formed cholic acid and elevated plasma levels of cholesterol when the amount of cholic acid in enterohepatic circulation is increased. No changes in the extent of conversion of labeled cholesterol to cholic acid or in plasma concentrations of cholesterol were seen in the animals receiving daily supplements of unlabeled cholesterol. Increase of hepatic cholesterol in this group may be due to an accumulation of dietary cholesterol which could not be compensated by suppression of hepatic biosynthesis demonstrated by Siperstein and Fagan (2). This control of cholesterol biosynthesis operates by inhibiting the reduction of β-hydroxy-β-methylglutarate to mevalonate in the liver and it is triggered by high ingestion of cholesterol. It is also known that this mechanism functions only when cholesterol is absorbed via the intestinal tract (6).

A role of cholic acid concerning physiologic or pathologic regulation of body cholesterol levels or both has been shown in mice (10) and is also suggested by the prolonged enterohepatic cycling of this compound in the guinea pig (13). Dietschy's (12) and present data indicate that this is also the case in the rat. The changes observed in animals with increased amount of cholic acid in the enterohepatic cir-

TABLE I

Plasma and Tissue Concentration of Cholesterol in Control Animals and After 15 Days of Daily Duodenal Administration of Cholesterol or Cholic Acid

Experimental group	Plasma mg/100 ml	Liver g/100 g	Kidneys g/100 g	Aorta g/100 g
Control	73.1 ± 8.6[a]	0.38 ± 0.03	0.41 ± 0.04	0.23 ± 0.01
Cholesterol	71.1 ± 10.2	0.69 ± 0.04	0.44 ± 0.07	0.29 ± 0.02
Cholic Acid	103.0 ± 11.2	0.36 ± 0.06	0.44 ± 0.09	0.25 ± 0.06

[a]Mean value ± 1 SD.

culation (Fig. 3) strongly suggest reduction of metabolic transformation of cholesterol to cholic acid and could serve as experimental models for diseases associated with hypercholesterolemia. Similar conditions exist in rats treated with intestinal antibacterial chemotherapeutics (21) and such animals show prolonged excretion of labeled cholic acid in feces (22).

The cholic acid molecule is normally dehydroxylated at the 7 position in the sterol nucleus by the action of bacteria in the intestinal tract of the rat. It is thus transformed into deoxycholic (3α,12α-dihydroxycholanic) acid. The compound is reabsorbed in this latter form and rehydroxylated in the liver before it is excreted (8). When animals are treated with antibacterial chemotherapeutics, intestinal bacterial growth is retarded, bacterial action is less effective or even abolished and dehydroxylation may not occur. In such case cholic acid is reabsorbed unchanged and possibly in larger quantity than its dihydroxy derivative because it is more polar. This sequence of events may explain the prolonged enterohepatic circulation of cholic acid associated with a possible alteration of the intestinal bacterial flora. Prolonged enterohepatic circulation of cholic acid is also observed in rats receiving cholic acid supplements (this study) and in patients with hypercholesterolemia (14). An excessive production of cholic acid by the liver may be responsible for the genesis of non-dietary hypercholesterolemia in these patients (14). Also, changes in the genetic control of cholesterol biosynthesis may be a contributory factor (23). High cholesterol levels in tissues and plasma may be followed by enhanced formation of bile acids. Dihydroxy acids may be rapidly excreted via the intestinal tract while cholic acid may be reabsorbed in large quantities and inhibit its own synthesis from cholesterol. Such inhibition may moreover lead to abnormal accumulation of cholesterol because its main path of excretion is blocked.

ACKNOWLEDGMENTS

This work was supported in part by grants I-GS-128 and AM-08941 from the National Institutes of Health.

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[Received June 6, 1968]

Fatty Acid Composition of Baboon Milk Lipids

DAVID H. BUSS, Division of Biological Growth and Development, Southwest Foundation for Research and Education, San Antonio, Texas 78206

ABSTRACT

The predominant fatty acids of mature baboon milk were shown by gas-liquid chromatography to be (by weight) palmitic (16%), oleic (23%), and linoleic (38%) acids. The high proportion of linoleic acid was attributed to the corn oil in the diet. Less myristic acid (1.3%), but more caprylic (5%) and capric (8%) acids, were present in baboon milk than in human milk. The proportions of these short-chain fatty acids were highest during early lactation.

INTRODUCTION

Although the fatty acid composition of human milk has long been known both from classical (1) and gas-liquid chromatographic (2) studies, only one report of the fatty acid composition of the milk of other primates has been published. Glass et al. (3) analyzed the lipids of two samples of milk from the green monkey, one from a rhesus monkey and one from a slow loris, but gave no details of the animals' diets. Baboon milk lipids have not been characterized, except for their cholesterol and phosphorus content (4).

Because baboons are widely used as experimental methods for man in many areas of health research (5,6) it is important that details of their pre-experimental diet be known. The present work describes the fatty acid composition of baboon milk, and relates this to the fatty acid composition of the diet and adipose tissue, and length of lactation of the animals.

EXPERIMENTAL PROCEDURES

Milk was obtained from 27 baboons (*Papio anubis, P. cynocephalus* and *P. papio*) by manual expression after sedation and treatment with oxytocin, as described elsewhere (7). No more than four samples were contributed by any baboon. All the animals were lactating normally, as judged by the health and rate of weight gain of their infants.

Lipids were extracted from the samples by the Röse-Gottlieb procedure as described by Walstra and Mulder (8), except that the combined extracts were washed once with water and concentrated to dryness under a stream of nitrogen. Three of the samples were also extracted by the procedure of Folch et al. (9), as was a piece of baboon depot fat taken from the retroperitoneum during a cesarean section. The methyl esters of the component fatty acids were prepared immediately by transesterification with sodium methoxide in a sealed tube; the modification of deMan's method (10) described by Storry et al. (11) was used.

The baboons were maintained on a diet consisting only of standard biscuits (12) and water ad lib. Fresh biscuits, and those which had been available to the baboons for a whole day at 27-36 C were ground separately in a Waring blendor for 15 sec; lipids were then extracted with petroleum ether (bp 40-60 C) for 16 hr in a Soxhlet apparatus under nitrogen. The extracts were concentrated to a small volume in vacuo and a portion of each, containing about 40 mg of lipids, was dried and transesterified as described above.

For chromatography, the contents of a transesterification tube were fractionated between petroleum ether (4 ml) and water (2 ml), the petroleum ether layer was concentrated to dryness under a stream of dry nitrogen, and 1.5-2 μliters of the methyl ester mixture were immediately injected into a Varian Aerograph model A90-P3 gas chromatograph fitted with a thermal conductivity detector. The column consisted of 15% diethylene glycol succinate polyester on chromosorb P (60-80 mesh, acid washed and silanized) in a 10 ft x 1/4 in. OD stainless steel tube. The helium flow rate was about 60 ml/min. The column temperature was programmed manually from 175-225 C at about 5 C min, then maintained at 225 C until all the esters had been eluted. The injector and detector were maintained at 250 C throughout.

The components were identified by comparisons with known mixtures of fatty acid methyl esters (Applied Science Laboratories Inc., State College, Pa.). Peak areas were masured with a Technicon integrator/calculator, and converted to weight per cent methyl ester by division with relative weight response factors, determined from quantitative standards (Applied Science Laboratories) under the operating conditions described above.

RESULTS AND DISCUSSION

The fatty acid composition of the feed and baboon depot fat are shown in Table I. The biscuits contain about 9% fat; 6% is added corn oil (12), which accounts for the high level of

TABLE I
Fatty Acid Composition of Baboon Feed and Depot Fat, Wt %

Fatty acid	Feed	Depot fat
14:0	0.5	1.7
16:0	12.8	20.5
16:1	0.5	1.9
18:0	3.8	4.0
18:1	25.3	32.2
18:2	50.2	37.2
18:3	2.4	1.0

[a]Unidentified broad peaks, in positions similar to methyl caprate and methyl laurate peaks, were present in the chromatogram from the fresh biscuits. They contributed 4.5% of the total area. The depot fat also contained 0.6% arachidic acid and 0.8% of another acid, tentatively identified as eicosenoic acid.

linoleic acid. Except for linolenic acid, the fatty acids in the feed were found to be stable throughout a hot day in a closed hopper, so the proportions of fatty acids ingested by the baboons have remained almost constant during the three years they have been fed this diet. The fatty acid composition of the adipose tissue reflected this dietary intake, as in humans (13).

The fatty acid composition of the milk at various lactation periods is summarized in Table II. With the experimental conditions used, the chromatographic peak for methyl arachidonate was too small to be reliably detected. The analytical results were the same whether the Röse–Gottlieb (8) or Folch (9) procedure was used to isolate the milk lipids. The results mainly show the composition of mature milk, because few samples could be obtained before two weeks or after four months of lactation. One sample was, however, obtained as late as eight months postpartum. There was little difference between the fatty acid composition of the earliest and latest samples of mature milk, although as lactation progressed the composition tended slightly towards that of the dietary and depot fat, especially from four months onward, when the infants were being weaned.

During the transition from colostrum to mature milk (7), the fatty acid composition of the milk least resembled that of the dietary and depot fat. This milk contained the largest quantities of short-chain fatty acids, especially caprylic and capric acids. Butyric acid was, however, never detected, although added methyl butyrate was readily observed. The proportions of oleic, linoleic and linolenic acids were also furthest from those in dietary fat during this transitional period, indicating that the lipid biosynthetic activity of baboon mammary glands was highest at this point.

Baboon milk fatty acids resembled those from the milk of rhesus and green monkeys (3) more than from human milk (1,2). Milk from these subhuman primates was rich in caprylic and capric acids, and poor in myristic acid compared with human milk, indicating the differences between the biosynthetic activities of human and subhuman primate mammary glands.

Linoleic acid was the major fatty acid in almost every sample of baboon milk analyzed, whereas in other primates palmitic and oleic acids predominate (1-3). This results from the high level of linoleic acid the baboons' diet and depot fat, for a high level of linoleic acid in the diets of lactating women causes linoleic (with linolenic) acid to be 43% of the milk fatty acids (14).

TABLE II
Fatty Acid Composition of Baboon Milk Lipids during Various Lactation Periods, Wt %

	Length of lactation			
Fatty acid	2 days (1)[a]	7 days (2)	14-113 days (48)	144-252 days (6)
6:0	0.1	0.2	0.37 ± 0.05[b]	0.2 ± 0.01
8:0	5.7	10.0	5.1 ± 0.4	3.7 ± 0.5
10:0	7.4	14.7	7.9 ± 0.6	7.3 ± 1.5
12:0	1.7	3.0	2.3 ± 0.2	3.0 ± 0.6
14:0	1.3	1.6	1.3 ± 0.1	1.4 ± 0.3
15:0	0.3	0.1	0.15 ± 0.02	0.3 ± 0.2
16:0	19.6	16.2	16.5 ± 0.4	16.1 ± 0.7
16:1	1.9	0.9	1.2 ± 0.1	0.9 ± 0.2
17:0	0.4	< 0.1	0.14 ± 0.02	0.1 ± 0.04
18:0	4.7	3.5	4.2 ± 0.1	3.6 ± 0.6
18:1	22.2	17.1	22.7 ± 0.4	23.5 ± 1.2
18:2	32.5	32.8	37.6 ± 1.1	38.6 ± 0.8
18:3	2.0	0.1	0.6 ± 0.1	1.5 ± 0.7

[a]Number of samples.
[b]Mean ± SE.

ACKNOWLEDGMENTS

The studies were supported by U.S. Public Health Service Grant GM-13252 from the Division of General Medical Sciences. Thanks are given to Grace Friedlander for technical assistance, and to Mary Williams for helpful discussions.

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[Received October 9, 1968]

Differences Between the Metabolism of Linoleic and Palmitic Acid: Utilization for Cholesterol Synthesis and Oxidation to Respiratory CO_2[1]

RICHARD J. CENEDELLA[2] and **ARTHUR ALLEN**, *Department* of Biochemistry
The Jefferson Medical College of Philadelphia, Philadelphia, Pennsylvania

ABSTRACT

Measurements were made of the incorporation of intragastrically administered 1-C^{14}-labeled linoleic and palmitic acid carbon into the total body cholesterol of the intact rat and of the rat's ability to oxidize these two labeled acids to respiratory CO_2. As compared with palmitic acid, the intact rat and isolated rat tissues exhibit a greater ability to metabolize linoleic acid. This is evidenced by a greater utilization of linoleic acid carbon for synthesis of total body cholesterol and also by a preference for the oxidation of linoleic acid. The greater incorporation of linoleic acid carbon into cholesterol appears to reflect the preferential oxidation of linoleic acid by the liver, a main site of fatty acid oxidation and cholesterol biosynthesis. This preference of the rat to utilize linoleic acid carbon for the synthesis of cholesterol may help to explain the well documented observation that the plasma cholesterol of the rat increases as the linoleic acid content of the diet increases.

INTRODUCTION

In recent years there has been much interest in differences between the metabolism of essential and nonessential fatty acids and particularly in relationships between the fatty acids and the metabolism of cholesterol. Considerable attention has been devoted to relationships between the composition of the dietary fatty acids and the cholesterol content of the plasma (1) and also the composition of the plasma cholesterol esters (1,2). Investigations of this area have revealed that in the rat plasma cholesterol levels increase as the linoleic acid content of the diet increases (1); whereas, in man the circulatory levels of cholesterol decrease in response to an increase in the polyunsaturated fatty acid intake (3). The reasons for these changes are largely unexplained. Since large amounts of acetyl CoA are formed during catabolism of fatty acids, it is conceivable that these substances contribute a significant portion of the total carbon required for the cholesterol biosynthesis and that differences between the degree to which fatty acids furnish carbon for cholesterol synthesis may partially explain the relationships between intake of a particular type of fatty acid and changes in the levels of body cholesterol. In an effort to investigate this possibility and to further study differences between the metabolism of essential and nonessential fatty acids, the present study measures the incorporation of labeled carbon from intragastrically administered 1-^{14}C-linoleic acid, an essential fatty acid, and 1-^{14}C-palmitic acid, a nonessential fatty acid, into the total body cholesterol of the intact rat. In addition, simultaneous measurements were also made on the extent of oxidation of these two labeled fatty acids to respiratory CO_2 in order to provide an index of the rates at which these two fatty acids were generating acetyl-CoA. A preliminary report on these experiments has been presented (4).

EXPERIMENTAL PROCEDURES

Three month old male rats of the Wistar strain, maintained on Purina rat chow, were fed by stomach tube, 1 ml of USP olive oil to which was added 12 μc of either 1-^{14}C-palmitic or 1-^{14}C-linoleic acid, having a specific activity of 1 μc/mg. Since the palmitic acid and linoleic acid content of USP olive oil is essentially identical (5), the specific activity of the 1-^{14}C-palmitate and 1-^{14}C-linoleate absorbed from the intestines should also be essentially identical. At various time intervals after administration of the labeled fatty acids, respiratory CO_2 was collected for a few hours (6) and the animals then killed. Rats were given access to their regular diet during the time period between administration of the labeled fatty acids and collection of respiratory CO_2.

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[1]This work is taken from a thesis by R. J. Cenedella in partial fulfillment of the requirements for the Ph.D. degree in biochemistry.

[2]Present Address: Department of Pharmacology West Virginia University Medical Center Morgantown, West Virginia 26506

TABLE I

Per Cent of the Total Absorbed 1-^{14}C-Palmitate and Linoleate Carbon Found Incorporated Into the Total Cholesterol of the Entire Rat, 6, 29 and 77 hr After Feeding 1-^{14}C-Palmitate or Linoleate[a]

Hours Killed	Number of rats	^{14}C-Cholesterol (^{14}C-Palmitate-fed rats)	Number of rats	^{14}C-Cholesterol (^{14}C-Linoleate-fed rats)
6	3	0.266 ± 0.024	3	1.252 ± 0.342
29	4	0.231 ± 0.030	4	0.804 ± 0.222
77	4	0.249 ± 0.067	4	0.800 ± 0.122

[a] Each value is expressed as the arithmetic mean ± 1 SD. The statistical significance of the differences between the incorporation of 1-^{14}C-palmitate and linoleate at each time studied is less than 0.01 (as measured by the Student's "t" test).

Unabsorbed lipid was collected by washing the entire gastrointestinal tract with acidic-aqueous-ethanol followed by petroleum ether and combining these washings with the excreted fecal matter. The unabsorbed lipid fraction and the entire rat were than saponified separately in alcoholic KOH and the fatty acids and colesterol extracted, essentially as described by Van Bruggen et al. (7). The isolated fatty acids were oxidized to CO_2 by wet combustion (8) and converted to baruim carbonate; the cholesterol converted to the digitonide (9), and the respiratory CO_2 precipitated as baruim carbonate. All ^{14}C-containing samples were plated to infinite thickness and counted with the Nuclear Model C110B detector. The total activity of all radioactive samples were calculated by multiplying the specific activity as baruim carbonate multiplied by the amount of carbon in the sample. The amounts of the ^{14}C fatty acids absorbed from the intestines were calculated by subtracting the total ^{14}C recovered in the fecal plus intestinal contents from the total ^{14}C fed. About 60% of both the fed 1-^{14}C-palmitate and linoleate was absorbed by the sixth hour after feeding; about 80% of each was absorbed by the twenty ninth and seventy-seventh hours. The percentage of absorbed ^{14}C fatty acid carbon incorporated into cholesterol in the rat was calculated directly by dividing the total activity of the cholesterol isolated from the whole rat by the total activity of the absorbed ^{14}C-labeled fatty acids multiplied by 100. The percentage of endogenous ^{14}C-labeled fatty acid carbon undergoing conversion to respiratory CO_2 per hour during various time intervals after feeding the labeled fatty acids was calculated as follows: the average-hourly total activity of the respiratory $^{14}CO_2$ collected during the 1 or 5 hr time intervals was divided by the total ^{14}C fatty acid activity recovered from the entire rat, minus intestinal contents, immediately after completion of the respiratory CO_2 collection. This value was then multiplied by 100.

Inasmuch as conversion of ^{14}C-labeled fatty acid carbon to cholesterol and respiratory CO_2 in some instances was measured several days after feeding of the labeled fatty acids, the question arose as to whether the endogenous ^{14}C-labeled fatty acids at such time were still essentially the same as the fed ^{14}C-labeled fatty acids. By chromatographic separation of the endogenous fatty acids into major classes (1), followed by ^{14}C assay (11), it was shown that the ^{14}C-fatty acids derived from the administered labeled palmitate and linoleate were present in the rat predominantly as saturated and diunsaturated fatty acid respectively even 7 days after the fatty acids were administered.

TABLE II

Per Cent of the Total Absorbed 1-^{14}C-Palmitate and Linoleate Carbon Found Incorporated Into the Total Cholesterol of Various Tissue Fractions 6 hr After Feeding These ^{14}C-Labeled Fatty Acids[a]

Fatty acid fed	Number of rats	Carcass[b]	Liver	Gut[c]	Skin[d]	Organs[e]
1-^{14}C-Pal.	3	0.058±0.015	0.085±0.020	0.055±0.025	0.050±0.027	0.017±0.004
1-^{14}C-Lin.	3	0.325±0.147	0.472±0.161	0.208±0.050	0.132±0.071	0.108±0.046

[a] Each value is expressed as the arithmetic mean ± 1 SD.
[b] Carcass refers to the tissue fraction remaining after removal of the liver, gut, skin and organs.
[c] Gut refers to the gastrointestinal tract from the stomach to the anus inclusive.
[d] Skin refers to the entire hide, ie., subcutaneous fat, skin and hair.
[e] Organs refers to the heart, spleen, pancreas, kidneys, adrenals and reproductive system.

TABLE III

Per Cent of the Total Endogenous ^{14}C-Labeled Fatty Acid Carbon Oxidized to Respiratory $^{14}CO_2$ Per Hour by the Intact Rat During Various Time Intervals After Feeding 1-^{14}C-Palmitate or Linoleate[a]

Hours after feeding	Number of rats	$^{14}CO_2$ (^{14}C-Palmitate-fed rats)	Number of rats	$^{14}CO_2$ (^{14}C-Linoleate-fed rats)
5--6	4	10.88 ± 2.99	4	20.94 ± 7.16
24--29	5	2.11 ± 0.51	5	2.43 ± 0.59
48--53	3	0.94 ± 0.10	3	0.79 ± 0.22
72--77	4	0.75 ± 0.49	4	0.80 ± 0.20
168--173	1	0.59	1	0.67

[a] Each value is expressed as the arithmetic mean ± 1 SD. The difference between the oxidation of 1-^{14}C-palmitate and linoleate during the 5 to 6 hr time interval is significant to the 0.05 level (as measured by the Student's "t" test). The differences at the other time intervals are not statistically significant.

RESULTS

The results shown in Table I indicate that at 6, 29 and 77 hr after feeding of the labeled fatty acids, a significantly greater fraction of the absorbed ^{14}C-linoleate than ^{14}C-palmitate carbon was recovered as ^{14}C-cholesterol in the entire rat. For example, after one or three days, about 0.8% of the absorbed ^{14}C-linoleate carbon was present as cholesterol compared to about 0.2% of the absorbed ^{14}C-palmitate. In several additional experiments it was demonstrated that this preferential incorporation of administered linoleate carbon into cholesterol in the entire rat was occurring in all of a large variety of tissues examined following the feeding of the labeled fatty acids (Table II).

The observed differences between the fraction of absorbed linoleate and palmitate carbon converted to cholesterol appears to be the result of events that occurred soon after the labeled fatty acids were absorbed, since as shown in Table I, the concentration of ^{14}C in the cholesterol of the rats fed either labeled palmitate or linoleate remained essentially constant between one and three days after administration of the fatty acids.

The explanation for the preferential incorporation of absorbed linoleate carbon into cholesterol appears related to the observation that there was an accompanying preferential oxidation of ^{14}C-labeled linoleate compared to ^{14}C-labeled palmitate shortly after administration of these fatty acids. This is shown in both Tables III and IV. Between the fifth and sixth hours after feeding the labeled fatty acids, approximately twice as much endogenous ^{14}C-fatty acid carbon was oxidized to CO_2 per hour by the intact, 1-^{14}C-linoleate-fed rats than by the intact, 1-^{14}C-palmitate-fed rats (Table III). In addition, when removed from the rats 6 hr after feeding and incubated in vitro, both the liver and skeletal muscle of these animals showed a preference for oxidation of endogenous linoleate (Table IV). As further equilibration of the labeled fatty acids with the body

TABLE IV

Per Cent of the Endogenous Tissue ^{14}C-Labeled Fatty Acid Carbon Oxidized to Respiratory $^{14}CO_2$ Per Gram of Tissue (Wet Weight)[a,b]

Hours after feeding	Number of rats	$^{14}CO_2$ (^{14}C-Palmitate-fed rats)		Number of rats	$^{14}CO_2$ (^{14}C-Linoleate-fed rats)	
		Liver	Muscle		Liver	Muscle
6	4	1.43 ± 0.41	8.67 ± 2.50	4	2.82 ± 0.23	16.61 ± 3.63
29	2	1.07 ± 0.07	2.29 ± 0.10	2	1.13 ± 0.01	2.83 ± 0.03
77	4	1.24 ± 0.08	1.62 ± 0.56	4	0.98 ± 0.06	2.22 ± 0.83

[a] At 6, 29 and 77 hr after feeding the ^{14}C-labeled fatty acid 1 g samples of liver and skeletal muscle were removed, sliced (Stadie tissue slicer) and incubated in 20 ml of Krebs phosphate buffer (pH = 7.4) for 2 hr at 37 C.

[b] Each value is the mean ± 1 SD. Significantly more endogenous ^{14}C from 1-^{14}C-linoleate than 1-^{14}C-palmitate was oxidized to $^{14}CO_2$ by both liver and muscle at the sixth hour, P (t) $<$ 0.02(as measured by the Student's "t" test).

lipid occurred over a period of a day or more, the fraction of endogenous ^{14}C-fatty acid carbon oxidized to CO_2 per hour in the linoleate-fed rat was similar to that observed in the palmitate-fed rat (Tables III and IV).

DISCUSSION

Since the liver is a primary site of oxidation of fatty acids recently absorbed from the intestines and also a main site of cholesterol biosynthesis, the observed preferential utilization of dietary 1-^{14}C-linoleate carbon for cholesterol biosynthesis by the intact rat could largely represent a preferential catabolism of 1-^{14}C-linoleate by the liver. Indeed, the data indicate that soon after ingestion of 1-^{14}C-linoleic and palmitic acids the liver was oxidizing significantly more 1-^{14}C-linoleic acid carbon than 1-^{14}C-palmitic acid carbon. The greater oxidation of the fed 1-^{14}C-linoleate than 1-^{14}C palmitate by the liver perhaps could reflect a smaller pool of hepatic linoleic acid available to dilute the newly absorbed ^{14}C-linoleate than hepatic palmitic acid to dilute the ^{14}C-palmitate. It is also possible that the enzymes of the β oxidation pathway exhibit a preference for linoleic over palmitic acid as substrate for oxidation.

Irrespective of the mechanism involved, as judged by ability to oxidize linoleic and palmitic acids and to utilize the carbon of these acids as substrate for cholesterol synthesis, the rat exhibits a greater ability to catabolize linoleic than palmitic acid. The greater incorporation of linoleic acid carbon into cholesterol most probably reflects the preferential oxidation of linoleic acid by the intact rat and by the rat liver in particular. Although utilization for cholesterol biosynthesis is a quantitatively minor pathway of fatty acid metabolism, the fact that animals ingest considerable amounts of fatty acids and that cholesterol represents a small fraction of the total body carbon makes it likely that a large fraction of the carbon required for total cholesterol synthesis is derived from fatty acid carbon. Thus, the observed preference of the intact rat to utilize linoleic acid carbon for the synthesis of cholesterol may help to explain the observation of Klein (1) and others (12) that the plasma cholesterol of the rat increases as the linoleic acid content of the diet increases. By comparison, the decrease in plasma cholesterol of humans in response to increased intake of linoleic acid may relate to the observation of Fredrickson and Gordon (13) that the human does not catabolize linoleic acid as readily as palmitic and oleic acids.

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[Received October 14, 1968]

GLC Analysis of *Heliothis virescens* Triglycerides at Various Metamorphic Stages

RANDALL WOOD, R. D. HARLOW Medical Division[1] Lipid Research Laboratory, Oak Ridge Associated Universities, Oak Ridge, Tennessee 37830 and **EDWARD N. LAMBREMONT**[2] Nuclear Science Center, Louisiana State University, Baton Rouge, Louisiana 70803

ABSTRACT

The triglycerides of *Heliothis virescens* (F.), the tobacco budworm, reared on a semidefined diet, were examined at different stages of development. The distribution of triglyceride carbon numbers and the composition of triglyceride fatty acids were determined by gas-liquid chromatographic analysis of intact triglycerides and methyl esters. Triglycerides of carbon number 50 decreased in the unfed adults with age; the decrease corresponded with a decrease in palmitic acid. Linoleic acid and carbon number 50 exhibited fluctuations in concentrations that were correlated with metamorphosis. The observed changes in specific triglyceride carbon numbers with a simultaneous change in specific triglyceride fatty acids indicate a nonrandom fatty acid distribution in the triglycerides of this insect.

INTRODUCTION

Insects contain a high percentage of lipid, a large amount of which is triglyceride that serves as a source of energy (1). Although the fatty acid content of the triglyceride fraction has been analyzed for several insects (2,3), the intact triglyceride structure has not yet been examined for any insect. This communication describes the distribution of triglyceride carbon numbers and the fatty acid composition of triglycerides found in the tobacco budworm, *Heliothis virescens* (F.), during the larval to adult transformation, and in unfed adults of various ages.

EXPERIMENTAL PROCEDURES

Insect Material

Heliothis virescens (F.) (Lepidoptera: Noctuidae), the tobacco budworm, was reared in individual plastic containers from the egg to adult stage on a semidefined diet described by Berger (4). Larvae were removed from the diet and held 8 hr without food prior to extraction of lipids to minimize dietary triglyceride present in the intestinal tract. Late-stage pupae were placed in screen-topped containers of sawdust until moths emerged. Adults were placed in screen-topped half-gallon jars and given only water in order to deplete their lipid content.

Last instar larvae, pupae, and adults were homogenized in ice-cold water, lyophilized, and the total lipids were extracted by the Folch et al method (5). Neutral lipids were separated from phospholipids by silicic acid chromatography (6). Triglycerides were isolated by thin-layer chromatography (TLC) and hydrogenated before analysis by gas-liquid chromatography (GLC). Methyl esters were prepared from the triglycerides by transesterification with 2% sulfuric acid in methanol.

Thin-Layer Chromatography

Silica Gel G adsorbent layers (250 μ) were spread on 20 x 20 cm glass plates with a modified Colab applicator (7). Chromatoplates were air-dried, activated 30 min at 110 C, and stored in a desiccator until used. Neutral lipids or total lipids were applied to the plate in a narrow band with a Rodder Streaker (Rodder Instrument, Los Altos, Calif.). Solvent development was carried out in hexane-diethyl ether (90:10 v/v). The triglyceride band was located by spraying the chromatoplate lightly with a 0.2% Rhodamine 6G in 95% ethanol. The triglyceride band was scraped into a fritted glass funnel and eluted from the adsorbent layer with several volumes of diethyl ether.

Gas-Liquid Chromatography

Methyl esters and intact triglycerides were analyzed with an Aerograph Model 204 gas chromatograph. One side of the dual column instrument was modified for the analysis of triglycerides and other high molecular weight compounds. The modifications will be described in a separate publication. Triglycerides were analyzed on a 70 cm x 4 mm OD (2.5 mm ID) Pyrex column packed with 1% OV-1 on 100-120 mesh Gas Chrom Q manually temperature programmed from 200-335 C at approxi-

[1]Under contract with the U. S. Atomic Energy Commission.

[2]Oak Ridge Associated Universities Research Participant.

TABLE I

Lipid Content of *Heliothis virescens* at Various Metamorphic Stages

Metamorphic stage	Number of insects	Lipid, %		Lipid, %	
		Dry wt.	Wet wt.	NL[a]	PL[b]
Young larvae	66	15.2	2.7	92.7	7.3
Mature larvae	40	14.2	9.4	92.3	7.7
Young pupae	42	43.3	14.9	93.7	6.2
Mid-age pupae	50	26.5	9.2	96.4	3.6
Old pupae	39	26.4	8.3	91.0	9.0
Newly emerged adults	42	29.8	12.7	94.7	5.3
4 Day old adults	22	27.4	11.6	--	----
7 Day old adults	26	15.0	6.4	--	----
11 Day old adults	15	9.4	3.0	--	----
Larval diet	---	2.6	0.6	76.1	23.9

[a]NL, neutral lipid.

[b]PL, phospholipid.

mately 5 C/min. Temperatures of the flash heater and detector were maintained at 325 C and 350 C. Helium carrier gas, hydrogen and oxygen flow rates were 100, 50, and 300 ml/min, respectively.

Methyl esters were chromatographed on a 152 cm x 3 mm OD (1.75 mm ID) Pyrex column packed with 15% ethylene glycol succinate silicone polymer (EGSS-X) coated on 100-120 mesh Gas Chrom P. The column temperature was manually programmed from 125-190 C at approximately 3 C/min. Flash heater and detector temperatures were maintained at 250 C. The flow rate of the helium carrier gas was 40-60 ml/min. Hydrogen and air flow rates were regulated to give maximum detector sensitivity.

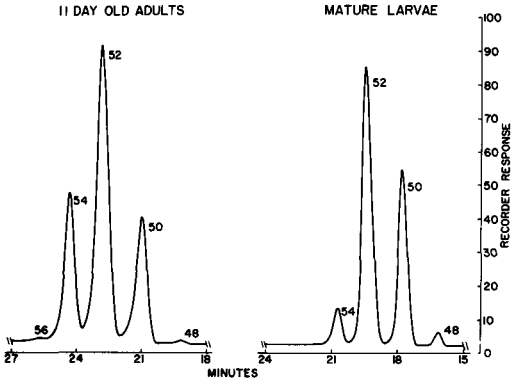

FIG. 1. Representative chromatograms (GLC) of intact triglycerides of *Heliothis virescens* larvae and adults. The comparison illustrates the changes that occur in the carbon number distribution of the triglycerides during development. Chromatographic conditions are described in the text.

Methyl ester and triglyceride peak areas were measured by triangulation and represent the mean of three determinations. Methyl ester values are given as uncorrected area percentages since values obtained for NIH standard methyl ester mixtures (D and F) agreed (±5%) with the known percentages. Standard triglyceride mixtures were used for instrument calibration and the mole percentages given have been corrected for slight losses of the higher molecular weight triglycerides.

Materials

Purified triglycerides and methyl ester standard mixtures were obtained from The Hormel Institute, Austin, Minn. Glass-distilled solvents were purchased from the Burdick and Jackson Laboratories, Inc., Muskegon, Mich. Other chemicals were reagent grade or better and were used without further purification.

RESULTS AND DISCUSSION

The percentages of total lipids, neutral lipids and phospholipids in *Heliothis virescens* at various stages of development are shown in Table I. The total lipid content, based on either wet weight or dry weight, shows the same trend: a maximum is reached at the early pupal stage and decreases to a minimum value in mid-age pupae. The total lipids remain constant until emergence of the adult, at which time the lipid content begins to decrease with age. The neutral lipids of *Heliothis* represent approximately 93% of the total lipids and appear to be unaffected by metamorphosis. Although the lipid class composition was not quantitatively determined, the neutral lipids consist primarily of

TABLE II
Calculated and Determined Triglyceride Carbon Number Distribution of *Heliothis virescens* at Various Stages of Development

Stage examined	Carbon number, %[a]					
	48	50	52	54	56	58
Young larvae	0.9[b]	17.9	64.3	16.5	0.5	----
Calculated[c]	4.4	22.3	42.1	29.1	3.8	0.2
Mature larvae	2.6	33.6	56.6	7.2	----	
Calculated	8.6	31.4	40.6	18.4	0.7	
Young pupae	2.3	31.7	58.6	7.4	----	
Calculated	8.1	30.0	39.6	18.2	0.7	
Mid-age pupae	2.5	35.2	51.4	10.4	0.5	
Calculated	8.8	31.3	39.6	18.5	1.5	
Old pupae	2.0	35.8	54.8	8.3	T	
Calculated	8.8	30.4	39.9	19.2	1.5	
Newly emerged adults	2.3	36.2	54.4	7.2	----	
Calculated	9.3	32.2	39.8	17.7	1.0	
4 Day old adults	0.8	21.6	62.2	15.4	----	
Calculated	5.5	24.7	42.0	23.8	1.8	
7 Day old adults	1.2	23.3	55.3	19.9	0.2	
Calculated	4.5	22.5	42.3	27.5	2.0	
11 Day old adults	0.8	20.2	53.5	25.3	0.2	----
Calculated	3.1	16.5	35.4	32.5	10.8	1.5
Larval diet triglycerides	0.4	7.9	39.2	48.2	3.6	0.6
Calculated	1.2	9.4	34.6	50.6	4.3	0.1

[a] The carbon number represents the sum of the number of carbon atoms in the hydrocarbon chains.

[b] Percentages represent the mean of three determinations.

[c] The random distribution percentages were calculated from the respective fatty acid percentages in Table III.

triglycerides.

Representative chromatograms of triglycerides isolated from mature larvae and 11 day old adults (Fig. 1) illustrate the change in carbon number distribution during development. Triglyceride carbon number distribution percentages obtained for different developmental stages and the diet are given in Table II along with calculated random distribution percentages. The fatty acid distribution in the triglycerides is nonrandom at all stages: determined percentages of carbon numbers 52 and 54 are 15-20% higher and 7-10% lower, respectively, than calculated values. The distribution of carbon numbers in this insect is very narrow (48-56) at all stages of development, with carbon numbers 50, 52 and 54 representing more than 95% of the total. After the young adult stage, the percentage of carbon number 50 decreased with age. Carbon number 54 increased but showed fluctuations corresponding to metamorphic changes. Following the early last instar larval stage, carbon number 52 showed the least variation. The difference between the carbon number distribution of dietary triglycerides and the insect triglycerides indicates the presence of an active triglyceride synthesizing system involving either total de novo synthesis, hydrolysis and rearrangement of dietary triglycerides, or a combination of both.

Fatty acid methyl esters of triglycerides ranged in chain length from $C_{14:0}$ to $C_{20:4}$ but C_{16} and C_{18} fatty acids represented more than 95% of the total in all stages of development (Table III). The dietary fatty acid composition (Table III) is not reflected in the fatty acid composition of the insect triglycerides. Oleic and linoleic acids represented 16% and 53% of the total dietary fatty acids, whereas these same acids represented 40-45% and 7-17%, respectively, of the triglycerides in *Heliothis virescens*. Our data are similar to those reported by Schaefer (8) who has reported that larvae of the bollworm, *Heliothis zea* (Boddie), when reared on wheat germ diet high in linoleic acid, contain only small amounts of this acid in the total lipids. As seen in Table III, the diet contained only traces of palmitoleic acid but the insect triglycerides contained up to 10% of this fatty acid. Vanderzant (9) and Schaefer (8) reported that palmitoleic acid is synthesized by *Heliothis zea* when this acid is absent from the diet. Oleic and palmitoleic acid percentages showed the least variation from early last instar

TABLE III
Triglyceride Fatty Acid Composition of
Heliothis virescens at Various Stages of Development

Metamorphic stage	Fatty acid, %[a]							
	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4
Young larvae[b]	0.3	31.7	2.5	2.1	42.5	17.0	1.5	3.1
Mature larvae[b]	0.4	33.9	9.2	0.8	47.6	6.7	0.6	0.8
Young pupae	0.3	33.8	8.6	0.9	45.8	7.9	0.8	0.7
Mid-age pupae	0.4	34.8	8.7	0.8	40.3	12.0	1.3	1.7
Old pupae	0.5	33.9	8.6	0.6	42.5	11.5	0.7	1.6
Newly emerged adults	0.5	34.4	9.7	0.7	43.8	9.9	T	1.1
4 Day old adults	0.4	29.9	6.2	0.3	46.3	14.6	0.6	1.6
7 Day old adults	0.5	26.7	7.0	0.6	46.6	16.0	1.0	1.6
11 Day old adults	0.5	23.3	6.1	0.7	42.5	16.6	1.3	9.0
Diet lipids	0.7	21.3	T	1.4	16.1	52.9	6.9	0.8
Diet triglycerides	0.7	18.5	T	1.5	18.9	51.7	6.5	2.3

[a] Percentages represent means of duplicate analyses.
[b] Last larval instar.

larval stage to the adult stage. Palmitic acid showed a decrease with age, and the same trend as observed for triglyceride carbon number 50. Linoleic acid exhibited fluctuations in concentrations corresponding to metamorphosis that was also observed for triglyceride carbon number 54.

The observed changes in specific triglyceride carbon numbers with a corresponding simultaneous change in specific triglyceride fatty acids and the lack of agreement between determined and calculated carbon number percentages indicate a nonrandom fatty acid distribution pattern. Adults (11 day old) maintained on water to deplete their lipid reserves (Table I) conserve arachadonic acid (Table III); this acid may serve a vital function in insects.

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[Received August 7, 1968]

SHORT COMMUNICATION

Incorporation of ^{14}C-UDP-Glucose and ^{14}C-UDP-Galactose Into Carbohydrate-Containing Sphingolipids by a Rat Brain Particulate Fraction

The biosynthetic pathway leading to the formation of galactosylceramide has been described. The initial reaction has been reported to be the glycosylation of sphingosine by UDP-galactose to yield galactosyl sphingosine (1). A particulate system has been reported which transfers the fatty acid moiety from a fatty acyl CoA derivative to the amino group of psychosine to produce galactosyl ceramide (2). Recently a brief publication appeared which reports the formation of glucosyl ceramide through the reaction of UDP-glucose and ceramide (3). The purpose of this communication is to describe the sphingosine stimulated incorporation of both UDP-glucose and UDP-galactose by a particulate fraction of rat brain. The product with UDP-glucose was found to be glucosylceramide while in the case of UDP-galactose it was galactosylsphingosine.

^{14}C-UDP-glucose and ^{14}C-UDP-galactose were obtained from New England Nuclear Corporation (Boston, Massachusetts). DL-erythro-sphingosine was purchased from Miles Laboratories (Elkhart, Indiana). ^{14}C-glucosyl and ^{14}C-galactosyl sphingosines as well as the unlabelled material were synthesized by a published procedure (4). The particulate preparation utilized as the enzyme source was similar to that reported by Cleland and Kennedy (1). Brains from 10 day old Sprague-Dawley rats were removed and homogenized in four volumes of 0.25 M sucrose -0.001 M EDTA pH 8.0. The suspension was centrifuged at 12,000 x *g* for 15 min and the supernate removed. The pellet was homogenized in the same solution in a volume equal to that removed and the suspension again spun at 12,000 x *g* for 15 min. The combined supernates were centrifuged at 30,000 x *g* for 1 hr. The pellet was suspended in 10 ml of 0.2 M Tris-0.00 M EDTA pH 8.0, and used as the enzyme source. Each incubation mixture contained 120,000 counts of either ^{14}C-UDP-glucose or ^{14}C-UDP-galactose, 2 μmole $MgCl_2$, 100 μg Tween-20, 20 μmole Tris-HCl pH 8.4 and 3 to 4 mg of enzyme protein in a total volume of 0.5 ml. The incorporation into lipids was assayed by a modified Folch partitioning procedure followed by a saponification treatment as described previously (5). Radioactivity was determined in a Nuclear Chicago gas flow proportional counter.

Thin-layer chromatography was carried out with Silica Gel G employing either borate impregnated or non-impregnated plates with chloroform-methanol-water (65:25:4 v/v/v) (6,7). Chloroform-methanol-water-15N NH_4OH (280:70:6:1 v/v/v) was used with borate plates to differentiate between glucosyl and galactosylceramide as well as between glucosyl and galactosyl-sphingosine. Radioactive tracings were obtained with a Bertholet TLC scanner.

Initial experiments designed to establish the subcellular distribution of the enzyme systems involved in the conversion of both nucleotide sugars revealed that nearly all of the activity resided in the particles brought down from 12,000 to 30,000 x *g*. Virtually no activity was obtained in either 30,000 x *g* supernatant or in the particles obtained at 0-12,000 x *g*.

The data in Table I document the ability of the particulate preparation to incorporate either ^{14}C-glucose (Part A) or ^{14}C-galactose (Part B) from their corresponding nucleotide derivative into alkali-stable lipid.

It is apparent that erythrosphingosine stimulates the incorporation of both sugars from their nucleotide derivative. The effect with UDP-galactose is more marked. Ceramide added in either chloroform-methanol, or as a sonicated emulsion did not stimulate the incorporation of sugars into lipid. Under conditions where there was an active stimulation of sugar incorporation, the presence of 1 μmole of the reciprocal non-radioactive sugar nucleotide did not depress the amount of radioactivity found in the lipid extract. Little if any effect was obtained by the presence of either glucosyl or galactosylsphingosine on the incorporation of ^{14}C-UDP-glucose. The presence of galactosylsphingosine did inhibit the conversion of ^{14}C-UDP-galactose into lipid.

The specific inhibition of ^{14}C-UDP-galactose incorporation by galactosylsphingosine without any effect of UDP-glucosyl sphingosine on ^{14}C-glucose incorporation suggested a difference in the conversion of these two sugars into lipid. This was corroborated by resolution of the products of the reaction by TLC. As shown in Figure 1A, ^{14}C-UDP-glucose in the absence of sphingosine gives rise to material which migrates with cerebroside (lane 1, tracing 1).

In the presence of sphingosine no change in the reaction product is seen (lane 2, tracing 2). In a similar manner, ^{14}C-UDP-galactose in the absence of sphingosine also gives rise to a cerebroside (lane 3, tracing 3) as shown in Figure 1B. In the presence of sphingosine, however, ^{14}C-UDP-galactose is preferentially incorporated into psychosine (lane 4, tracing 4). By using TLC with borate impregnated plates in systems which have been documented to

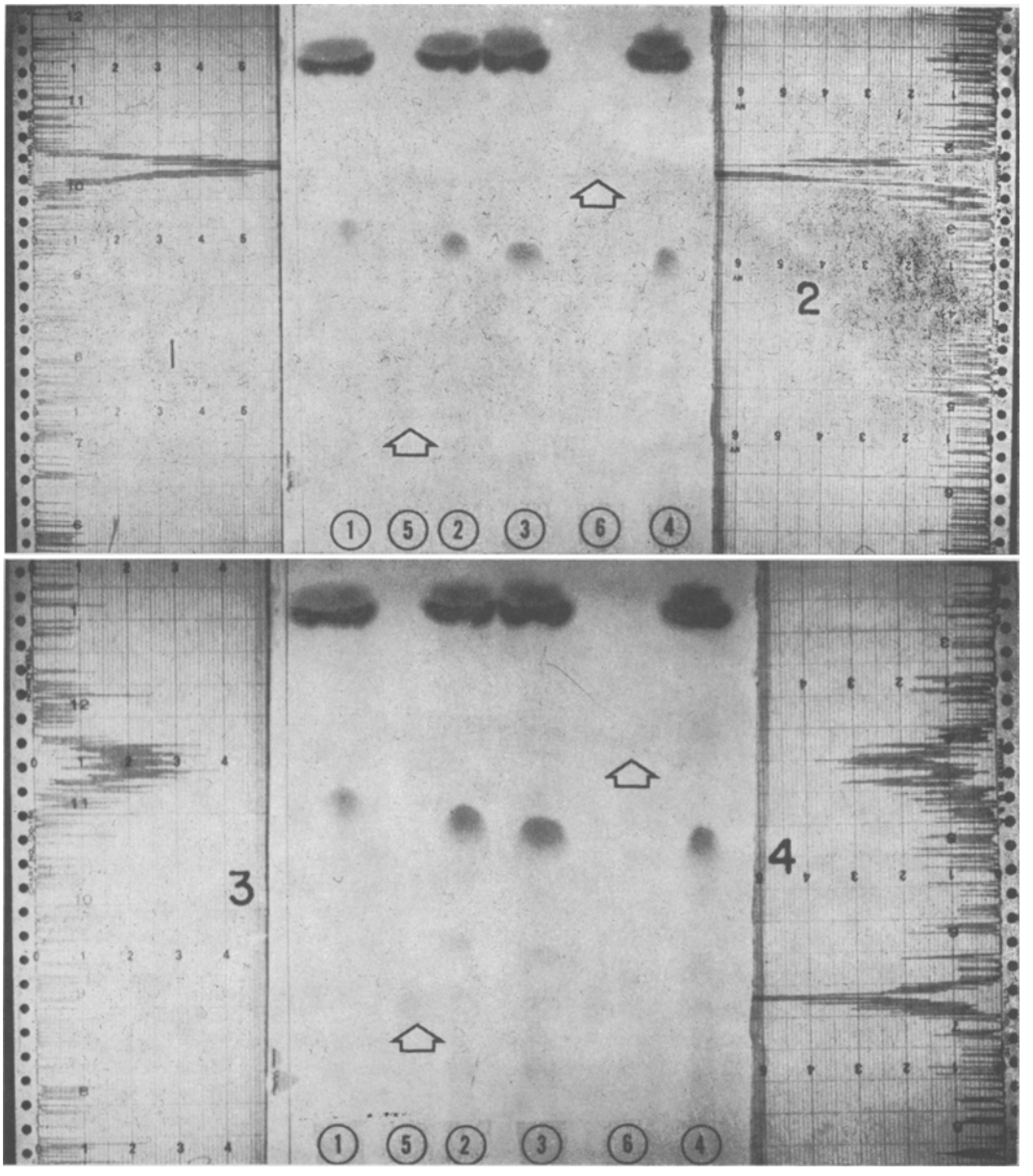

FIG. 1A and 1B. TLC of lipid extracts prepared as described in the text on Silica Gel G without borate in chloroform-methanol-water (65:25:4 v/v/v). The arrows indicate the position of spots which were clearly visible on the TLC plate but which were faint in the photograph. Lane 1, incubation of ^{14}C-UDP-glucose alone; Lane 2, incubation of ^{14}C-UDP-glucose + sphingosine; Lane 3, incubation of ^{14}C-UDP-galactose alone; Lane 4, incubation of ^{14}C-UDP-galactose + sphingosine; Lane 5, glucosylsphingosine standard; Lane 6, glucosylceramide standard. Tracing 1, obtained from lane 1; Tracing 2, obtained from lane 2; Tracing 3, obtained from lane 3; Tracing 4, obtained from lane 4.

TABLE 1
Effect of Various Acceptors on the Conversion of ^{14}C-UDP-glucose and ^{14}C-UDP-galactose into Lipids

	Condition	cpm Incorporated
A.	^{14}C-UDP-glucose	
	No additions	1000
	+ Erythro-sphingosine (0.3 μmoles)	2220
	+ Threo-sphingosine (0.3 μmoles)	1050
	+ Ceramide (0.3 μmoles)	1170
	+ UDP-galactose (1 μmole) + erythro-sphingosine	3000
	+ Glucosylsphingosine (0.3 μmoles) + erythro-sphingosine	2400
	+ galactosylsphingosine (0.3 μmoles) + erythro-sphingosine	2000
B.	^{14}C-UDP-galactose	
	No additions	220
	+ Erythro-sphingosine (0.3 μmoles)	2450
	+ Threo-sphingosine (0.3 μmoles)	250
	+ Ceramide (0.3 μmoles)	380
	+ UDP-glucose (1 μmole) + erythro-sphingosine	2500
	+ Glucosylsphingosine (0.3 μmoles) + erythro-sphingosine	1800
	+ Galactosylsphingosine (0.3 μmoles) + erythro-sphingosine	300

distinguish between these two cerebrosides (6,7), it was demonstrated that the ^{14}C-UDP-glucose was incorporated into the glucosylceramide while the ^{14}C-UDP-galactose was incorporated into galactosylceramide. TLC of the reaction products formed in the presence of ^{14}C-UDP-glucose sphingosine and glucosyl or galactosylsphingosine revealed that glucosylceramide was the only radioactive material produced. By employing borate-impregnated thin-layer plates, it was demonstrated that the psychosine produced from ^{14}C-UDP-galactose was galactosylsphingosine. Experiments in which ^{14}C-glucosyl or ^{14}C-galactosyl sphingosine were incubated in the enzyme system revealed that these compounds were unaltered and not converted to cerebrosides. The presence of palmitoyl CoA did not alter the nature of the products formed nor increased the total radioactivity incorporated in any of the experimental conditions.

The experiments described demonstrate the ability of a particulate fraction of rat brain to incorporate either ^{14}C-glucose or ^{14}C-galactose into sphingoglycolipid from their nucleotide derivatives. The stimulation by sphingosine is most marked with ^{14}C-UDP-galactose yielding as the principal product galactosylsphingosine. In the case of ^{14}C-UDP-glucose the product is glucosylceramide. The nature of the endogenous acceptor is unknown at this time; however, a lipid extract of the particles did not result in any stimulation of sugar incorporation into sphingolipid. In addition, the inability of ceramide to stimulate in this system would suggest that this compound is not the acceptor. The finding that the addition of the alternate nonradioactive sugar nucleotide did not depress the conversion to lipid would suggest that UDP-galactose-4 epimerase activity was not present. Glucosylsphingosine neither inhibited the incorporation of ^{14}C-UDP-glucose in the presence of sphingosine nor changed the reaction product (glucosylceramide); therefore, it would appear that this material is not an intermediate in glucosylceramide biosynthesis. This was further substantiated by the recovery of ^{14}C-glucosyl-sphingosine unchanged from the reaction mixture. Therefore, it appears that in this system the pathways of biosynthesis of glucosylceramide and galactosylsphingosine are different. Studies are currently being undertaken to delineate these reactions.

In order to isolate and identify the reaction products, the incubation mixtures were increased 15 fold. The products after incubating either ^{14}C-UDP-glucose or ^{14}C-UDP-galactose were isolated on separate silicic acid columns eluted in a stepwise fashion with increasing concentrations of methanol in chloroform. Carrier glucosylceramide was added to the product obtained by incubating ^{14}C-UDP-glucose and mixture crystalized from methanol. Constant specific activity was obtained after two recrystalizations. Carrier galactosylsphingosine was added to the product obtained by incubating ^{14}C-UDP-galactose. The mixture was treated with palmitoyl chloride. The reaction mixture was saponified to remove the esterified palmitic acid and galactosylceramide was isolated by silica acid column chromatography. Constant specific activity was obtained after three crystalizations. This material cochromatogramed with authentic standards, on borate-impregnated thin-layer plates, of galactosyl ceramide.

JULIAN N. KANFER[1]
Laboratory of Neurochemistry
National Institute of Neurological Diseases and Blindness
National Institutes of Health,
Bethesda, Maryland 20014

[1]Present address: Departments of Biochemistry and Medicine, Duke University Medical School, Durham, N. C. 27706.

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[Received June 9, 1968]

Isolation of an Unusual Positional Isomer of Hexadecenoic Acid From a Parasitic Leptospire

Microorganisms have been reported to synthesize branched and cyclic fatty acids as well as certain positional isomers of unsaturated fatty acids (1) which are not generally found in mammalian cells. Of the several possible isomers of hexadecenoic acid, the 9 isomer is the most common in nature. However, 11-hexadecenoic acid has been isolated as a minor component (~1%) of two bacteria, *Clostridium pasteurianum* and *Streptococcus hemolyticus* (1,2). In the current investigation of *Leptospira interrogans,* serotype *ballum* S102, 11-hexadecenoic acid was observed as a major lipid component (22.3%).

Cells were cultivated in 1 liter volumes in a bovine albumin medium (3) in which Tween 80 was replaced by 3 x 10^{-4} M hexadecanoic acid and 1 x 10^{-4} M 9-octadecenoic acid for 5 to 7 days at 30 C. These cells, which were in the late log or early stationery growth phase, were sedimented at 12,000 x *g* for 20 min and washed once with distilled water. Approximately 65 mg dry weight of cells were obtained per liter of medium and 450 mg dry weight of pooled cells were used for the lipid analyses. The lipid was extracted from lyophilized cells with chloroform-methanol (2:1) (4). Ten to 20 mg of total lipid were fractionated into neutral lipid and phospholipid by thin-layer chromatography (TLC) using microplates (5) developed with diethyl ether. For recovery of the lipid fractions, the silica gel containing the phospholipid and the neutral lipid was scraped into methanol and chloroform, respectively. Five to 10 mg each of total lipid, neutral lipid and phospholipid were saponified and acidified, and the free fatty acids extracted with hexane. Esterification of the fatty acids was performed with boron trifluoride (6) or diazomethane (7). Analysis of the methyl esters of the fatty acids was performed by gas-liquid chromatography (GLC). The C_{16} and C_{18} esters were collected separately by preparative GLC and analyzed by GLC. Each fraction was analyzed for purity and a small sample was hydrogenated to confirm the chain length by GLC. The individual samples were ozonized and reduced (8). The resulting aldehydeesters (ald-esters) derived from the splitting at the double bond were analyzed quantitatively by GLC.

TABLE I

Analysis of the C_{16} and C_{18} Fatty Acids From *Ballum* S102

	Total Percentage		
Fatty Acid	Total Lipid	Neutral Lipid	Phospholipid
Hexadecanoic	26.3	21.6	22.6
9-Hexadecenoic	3.0	5.1	2.8
cis-11-Hexadecenoic	22.3	20.6	28.9
Octadecenoic	1.0	2.8	0.4
9-Octadecanoic	37.6	43.1	25.4

The results are presented in Table I. The 11-hexadecenoic acid comprises 22.3% of the total fatty acids. This acid is the largest component of the phospholipid (28.9%), but it is also found as a major component in the neutral lipid (20.6%). The ozonized hexadecenoic esters were found to contain 89.7% C_{11} ald-ester and 10.3% C_9 ald-ester, showing the presence of both 9- and 11-hexadecenoic acids and the relative proportions of each. Infrared spectrophotometric analysis identified the 11-hexadecenoic acid as being the *cis* isomer. The amount of the 9-hexadecenoic acid available was insufficient for infrared spectrophotometric analysis. The ozonized octadecenoic acid fraction contained only the C_9 ald-ester demonstrating this acid to be entirely the 9 isomer.

Fatty acids are the major carbon and energy source for the leptospires (9). The parasitic leptospire, *ballum* S102, can grow optimally on unsaturated fatty acids with a minimum chain length of C_{16} (10). Saturated fatty acids cannot be utilized for growth unless an unsaturated fatty acid is also provided, in which case one of these fatty acids must be at least C_{16} (10). The pathway involved in the biosynthesis of *cis*-11-hexadecenoic acid in *Ballum* S102 is not known at this time.

BRIAN P. LIVERMORE
RUSSELL C. JOHNSON
HOWARD M. JENKIN[1]
Department of Microbiology
University of Minnesota
Minneapolis, Minnesota 55455

[1]University of Minnesota, The Hormel Institute, Austin, Minnesota 55912.

ACKNOWLEDGMENTS

The authors thank D. M. Sand for technical advice. This investigation was supported in part by research grants AI 06589 (NIAID) and HE 08214 (NHI) of the National Institutes of Health, USPHS.

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[Received September 25, 1968]

Silver Acetate for Stabilizing Methyl Galactosides After Methanolysis of Glycolipids[1]

A method for the characterization of glycolipids by GLC was reported by Sweeley and Walker (1) and successfully applied by Feldman et al. (2) and Vance and Sweeley (3). In this procedure the carbohydrate moiety is recovered as an equilibrium mixture of anomers of the methyl glycosides after hydrolysis in methanolic hydrochloric acid. The methyl glycosides are then further resolved by GLC of their trimethyl silyl (TMS) derivative.

When the acid hydrolysate contains methyl galactosides it is necessary to remove the HCl before the hydrolysate is blown dry with a stream of nitrogen. Failure to do so results in the transformation of methyl galactosides into unidentified analogs as evidenced by the occurrence of five peaks upon GLC instead of the usual three that represent α, β and γ galactosides. The degree of transformation is directly related to the rate at which the hydrolysate is evaporated (Table I). The two analog peaks are minimal with a very rapid evaporation rate, but at slower rates they become larger with a concomitant reduction in the size of the methyl-γ-galactoside peak. We were unable to achieve sufficiently rapid evaporation to completely prevent these changes.

The galactoside transformation is a serious source of error when glucose to galactose ratios are determined. The analog peaks are eluted with the glucoside peaks giving a false ratio. Sweeley and Walker (1) removed the HCl by passing the hydrolysate through a small column packed with Amberlite CG-4B. However, the resin is slightly soluble in methanol, contaminating the sample with a yellow residue. A volume of 25-30 ml of solvent is required to elute the methyl glycosides from the resin, thus

[1]Presented in part at the AOCS-AACC Meeting, Washington, D.C., March, 1968; Symposium on "Practical Applications of Chromatography in Lipid Analyses."

TABLE I.

Percentage Composition[a] of Methyl Galactoside Anomers in Anhydrous Methanolic HCl After Various Treatments

Anomer	Treatment of galactosides		
	Rapid evaporation	Slow evaporation	Silver acetate
γ	15.6%	2.2%	19.8%
α	58.3	60.4	55.4
β	20.2	17.1	24.8
Unknown 1	5.9	9.3	....
Unknown 2	....	11.0	....
Total	100.0	100.0	100.0

[a]Percentage values calculated directly from the number of counts obtained with an electronic peak integrator (CRS-100, Infotronics Corp., Houston, Texas).

TABLE II

Percentage Composition of Glucose and Galactose Anomers From two Oligosaccharides and a Ganglioside After Neutralization of the Methanolysate With Silver Acetate[a]

Ogilosaccharide	Sugar	Anomer	% Known	Gal/Glu Ratio
Lactose	Galactose	γ	19.1	0.993
		α	59.7	
		β	21.2	
	Glucose	α	74.0	
		β	26.0	
Melibiose	Galactose	γ	19.4	1.019
		α	59.8	
		β	20.8	
	Glucose	α	74.0	
		β	26.0	
Monosialoganglioside (Beef Brain)	Galactose	γ	18.7	1.859[b]
		α	59.3	
		β	22.0	
	Glucose	α	79.0	
		β	21.0	

[a]Calculated from integrator counts.

[b]Slightly low results due to insufficient silver acetate which led to some loss of γ-galactoside.

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adding to the contamination problem. Moreover, the column procedure is inefficient because of the time required to prepare the column, elute the sample and then evaporate the eluate. We wish to report an improved procedure for stabilizing the methyl galactosides.

This procedure is based on neutralization of the HCl in the hydrolysate with silver acetate. Chloride is precipitated as the silver salt leaving acetic acid which is readily evaporated under a nitrogen stream. In use, 250 mg of silver acetate are added to 3 ml of hydrolysate in 0.5N methanolic HCl and thoroughly mixed in a test tube. The mixture is allowed to react at room temperature for 2–3 min and then filtered through sintered glass of ultra fine porosity. The precipitate is washed on the filter three times each with 1 ml of methanol and the washings collected in the same tube as the original filtrate. The hydrolysate can then be evaporated to dryness, derivatized and analyzed by GLC. Three well resolved peaks are eluted without interference from the galactosidic transformation products (Table I).

The attainment of quantitatively correct glucose to galactose ratios can only be realized when the HCl is neutralized (Table II). Removal of the interference from the galactosidic transformation products eliminates the need for calculating correction factors and provides cleaner and more accurate chromatograms in less time than previously described methods.

A. STEWART WINDELER
GERALD L. FELDMAN
Dept. of Ophthalmology
Baylor University
College of Medicine
Houston, Texas 77025

ACKNOWLEDGMENTS

Support for this study was provided by Grant No. Q-203 from the Robert A. Welch Foundation, Houston, Texas; USPHS Grant NB-04277-07 from the National Institute of Neurological Diseases and Blindness, Bethesda, Md.; and the Moody Foundation, Galveston, Texas.

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[Received September 6, 1968]

Biosynthesis of Glycerides in Freshly Secreted Sow's Milk[1]

McCarthy and Patton (1) demonstrated the ability of freshly secreted ruminant milk to synthesize glycerides and other lipids when supplied solely with a free fatty acid. This system is also capable of dehydrogenating substantial quantities of stearic acid to oleic acid (2). Data have been presented indicating that microsome-like particles in the serum of freshly secreted milk accomplish this glyceride assembly (3). Discovery of this lipogenic principle in the milk of cows and goats has been a stimulus to research on milk fat metabolism. This system has, however, not yet been demonstrated in a monogastric animal. This is a preliminary report on the occurrence of such a system in sow's milk.

Milk samples were collected from sows which had been milked out completely 1 hr before sample collection. Oxytocin (40 IU) was administered intravenously to stimulate milk letdown. Skim milk was prepared as previously described (1). 1-^{14}C-Palmitic acid (10 $\mu c/\mu M$), dissolved in 40-80 μ liter of ethanol, was added to the milk and incubations were conducted at 37 C for various time intervals. Methods of lipid extraction (4), fractionation by thin-layer (4) and silicic acid column chromatography (5,6), and ^{14}C assay (1) were the same as those used by others. Methods used to fractionate and determine radioactivity by autoradiography in the lipids of

TABLE I

Incorporation of 1-^{14}C-Palmitic Acid Into Ester Lipids by Whole Milk, Cream and Skim Milk From Equivalent Quantities of a Single Sow Milk Sample

Enzyme medium	^{14}C recovered[a]	
	Neutral esters	Phospholipids
Whole milk	64,561	850
Heated whole milk[b]	476	746
Cream	8,250	3,123
Skim milk	90,466	1,697

[a] Added, 190,003 cpm; results expressed in cpm.
[b] Heated at 65 C for 15 min.

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adding to the contamination problem. Moreover, the column procedure is inefficient because of the time required to prepare the column, elute the sample and then evaporate the eluate. We wish to report an improved procedure for stabilizing the methyl galactosides.

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GERALD L. FELDMAN
Dept. of Ophthalmology
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TABLE II

Incorporation and Distribution of ^{14}C in Lipid Classes of Neutral Esters When 1-^{14}C-Palmitic Acid Was Incubated for 1 hr With Sow Skim Milk

Expt. No.	Total per cent incorporation	Per cent distribution of recovered ^{14}C in neutral esters		
		Triglycerides	Diglycerides	Cholesterol esters
1	25.0	93.8	4.8	1.4
2	26.3	92.8	4.5	2.7
3	27.3	97.1	2.3	0.6
4	25.0	96.0	3.0	1.0
5	27.2	96.8	3.0	0.2
Average	26.2	95.3	3.5	1.2

microsome-like particles were the same as those used previously (3).

Table I indicates the extent of incorporation of the labeled acid in 90 min into neutral ester and phospholipid classes in whole milk and its component cream and skim milk fractions. The amount of activity incorporated into neutral esters, that is, triglycerides, diglycerides, monoglycerides and cholesterol esters, was impressive, particularly since no cofactors were added to the system. This study showed that the synthesizing activity was heat sensitive and also that skim milk incorporated greater quantities of the acid into neutral esters than did whole milk or cream. Cream was more effective in incorporating palmitate into phospholipids. Because of its activity, skim milk was used in all further experiments.

The per cent incorporation and distribution of ^{14}C in various neutral ester classes after 1 hr incubation with the labeled acid is shown in Table II. The per cent incorporation was quite consistent in all trials, with triglycerides containing by far the bulk of the activity, followed by diglycerides. There were small but significant quantities of ^{14}C in cholesterol esters. Monoglycerides contained only negligible quantities of radioactivity. Similar results were obtained with other sows of different breeds.

Table III contains data on the incorporation of palmitic acid into ester lipids by skim milk over a period of time. Triglycerides increased in ^{14}C content over the entire incubation period; while the ^{14}C content of diglycerides was much lower and relatively stable throughout the time course. Radioactivity of phospholipids fluctuated throughout the experiment, indicating a possible dynamic role for these components in the synthesizing system. These results, which were duplicated in several trials, conform to the present view of milk fat synthesis that triglycerides are an accumulating final product with diglycerides serving as an intermediate in their formation (4).

TABLE III

The Progressive Incorporation of 1-^{14}C-Palmitic Acid Into Ester Lipids When Incubated With Sow Skim Milk

Time of incubation (min)	Total ^{14}C incorporated[a]			
	Triglycerides	Diglycerides	Cholesterol esters	Phospholipids
15	4,264	351	38	247
30	7,581	341	33	365
60	8,177	396	25	242
90	9,095	345	50	335

[a] Added, 33,462 cpm; results expressed in cpm.

It was critical to this study to determine if incorporation of palmitic acid into neutral esters represented true net synthesis or simply exchange of labeled acid with unlabeled acids of ester lipids. To examine this question, skim milk was incubated 90 min with ^{14}C-palmitic acid. After 45 min, the sample was split into two equal portions, 2 mg of unlabeled palmitic acid was added to one portion, and incubation was then continued. Results representative of three experiments are presented in Table IV. The ^{14}C content of triglycerides in the control portion increased throughout the incubation period, whereas triglycerides in the treated sample leveled and maintained the ^{14}C content they had at the time of treatment. This is the expected result if net synthesis was occurring. Exchange would have been indicated if radioactivity in triglycerides of the treated sample had declined, since chance would be favoring replacement by unlabeled fatty acids from the medium. If diglycerides were serving as intermediates in the net synthesis of triglycerides they should have decreased in radioactivity after treatment because of their transitory nature. Results in Table IV show that this did occur.

As a final check on the equivalence of this synthesizing system to that of ruminants, skim milk was incubated 90 min with labeled palmitic acid and then the microsome-like parti-

TABLE IV

Effect of a Mid-point[a] Addition of 2 mg of Unlabeled Palmitic Acid on the Incorporation of 1-^{14}C-Palmitic Acid Into Neutral Ester Lipids of Sow Skim Milk

Time of incubation (min)	Total ^{14}C incorporated[b]; control treated					
	Triglycerides		Diglycerides		Cholesterol esters	
30	3,525	—	191	—	12	—
45	6,803	—	434	—	15	—
60	8,929	6,665	326	297	41	39
90	9,098	6,772	261	186	42	56

[a] Immediately after the 45 min sampling.

[b] Added, 35,885 cpm; results expressed in cpm.

cles were sedimented as previously described (3). Autoradiograms obtained showed that the major incorporation of radioactivity into triglycerides occurred in the fraction rich in these lipoprotein particles. These results are identical to those obtained by Patton et al. (3) with freshly secreted milk synthesizing systems from cows and goats.

Since results presented herein were readily duplicated with sows of different breeds, they suggest that freshly secreted swine milk contains a well organized system for glyceride synthesis. Apparently this system contains all enzymes and cofactors necessary for net synthesis of glycerides. Although much further work is required to determine the relationship of this system to in vivo milk fat synthesis, the ease of obtaining this physiologically available enzyme source should facilitate the task. These data confirm the earlier work of McCarthy and Patton (1) and suggest that this glyceride synthesizing activity is widespread in freshly secreted milks.

T. W. KEENAN
V. F. COLENBRANDER
Food Sciences Institute
Department of Animal Sciences
Purdue University
Lafayette, Indiana 47907

ACKNOWLEDGMENTS

S. Patton, R. D. McCarthy and P. S. Dimick gave assistance in preparation of the manuscript.

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[Received September 25, 1968]

The Structure of Alkane Diols of Diesters in Vernix Caseosa Lipids

Human skin is notorious for producing many types of fatty chains in the form of acids and alcohols (ref. 1 and 2 and others quoted there). Fatty chains with 2 OH groups (diols) have also been found in vernix caseosa in two independent investigations (3,4). Kärkkäinen et al. (4) reported the fatty portions of the diols as straight chain whereas Downing (3) reported them as branched but did not further identify them. Neither study located the two OH groups in the chains. In wool wax the OH groups are in the 1 and 2 positions (5-7) whereas in preen gland lipids, they are in the 2 and 3 positions (8). α-Hydroxy fatty acids and 1,2-diols of wool wax are configurationally related (6), and possibly arise from a common biosynthetic mechanism different from that of the 2,3-diols (8). We undertook this study to determine which of the two diol types, if either, human skin produced, and to determine whether the chains were straight or branched, and if branched, the type of branching.

Figure 1 outlines the preparation and analysis of the diols. The diols were obtained from the unsaponifiable part of material eluted where diesters should emerge (chromatogram I fractions 13 to 19). In diol diesters both OH groups are esterified with fatty acids. One would expect these diesters to emerge after elution of sterol esters plus wax esters (monoesters) but before elution of triglycerides (triesters). The intermediate polarity of fractions 13 to 19 relative to mono- and triesters is readily seen in TLC (Fig. 2a, b). Infrared spectra of these fractions has prominent carbonyl absorptions, no free OH absorptions and, in general, were very similar to spectra of monoesters of fatty acids with fatty alcohols.

Since fraction 15 was Liebermann-Burchard negative and showed only one spot by TLC, we saponified this fraction first and worked it up by our usual techniques. Although the separation of saponification products was incomplete and required additional separation (Fig. 1), only two types of substances were found: saponifiables with the same R_F as palmitic acid (Fig. 2d) and unsaponifiables with the same R_F as 1,2-diols (Fig. 2d) but not 2,3-diols (Fig. 2e.) Infrared spectra of the unsaponifiables also matched closely with synthetic straight chain 1,2-diols although some differences were noted in the 1400 to 1360 cm^{-1} region (discussed below). Thus, apparently fraction 15 was solely 1,2-diol diesters.

Fraction 13 gave a negative Liebermann-Burchard test when we removed material mi-

cles were sedimented as previously described (3). Autoradiograms obtained showed that the major incorporation of radioactivity into triglycerides occurred in the fraction rich in these lipoprotein particles. These results are identical to those obtained by Patton et al. (3) with freshly secreted milk synthesizing systems from cows and goats.

Since results presented herein were readily duplicated with sows of different breeds, they suggest that freshly secreted swine milk contains a well organized system for glyceride synthesis. Apparently this system contains all enzymes and cofactors necessary for net synthesis of glycerides. Although much further work is required to determine the relationship of this system to in vivo milk fat synthesis, the ease of obtaining this physiologically available enzyme source should facilitate the task. These data confirm the earlier work of McCarthy and Patton (1) and suggest that this glyceride synthesizing activity is widespread in freshly secreted milks.

T. W. KEENAN
V. F. COLENBRANDER
Food Sciences Institute
Department of Animal Sciences
Purdue University
Lafayette, Indiana 47907

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Since fraction 15 was Liebermann-Burchard negative and showed only one spot by TLC, we saponified this fraction first and worked it up by our usual techniques. Although the separation of saponification products was incomplete and required additional separation (Fig. 1), only two types of substances were found: saponifiables with the same R_F as palmitic acid (Fig. 2d) and unsaponifiables with the same R_F as 1,2-diols (Fig. 2d) but not 2,3-diols (Fig. 2e.) Infrared spectra of the unsaponifiables also matched closely with synthetic straight chain 1,2-diols although some differences were noted in the 1400 to 1360 cm^{-1} region (discussed below). Thus, apparently fraction 15 was solely 1,2-diol diesters.

Fraction 13 gave a negative Liebermann-Burchard test when we removed material mi-

TABLE I
Analysis of Alkane Diols of Vernix Caseosa Diesters

Peak No.	Per cent of total fraction	GLC[a] of diol acetonides[b] Carbon No.[c] based on 1,2-diol standard[d] (JXR)	1,2-diol standard[d] (EGSSX)	2,3-cis diol standard (EGSSX)	2,3-trans diol standard[e] (EGSSX)	GLC[a] of aldehydes from HIO_4 oxidation of diols[f,g] Carbon No.[c] based on (EGSSX)	Per cent of total
1	11.6	19.65	19.58	21.08	20.35	18.56	11.8
2	1.9	20.00	20.00	21.49	20.79	19.00	2.2
3	3.1	20.65	20.59	22.08	21.32	19.55	3.3
4	6.4	20.72	20.73	22.19	21.50	19.70	5.3
5	1.1	21.00	21.00	22.46	21.73	20.00	1.0
6	47.5	21.65	21.58	23.01	22.31	20.57	47.3
7	Trace	21.72 (?)	21.72 (?)	23.10 (?)	22.40 (?)	20.70 (?)	Trace
8	3.7	22.00	22.00	23.40	22.70	21.00	3.4
9	Trace	22.40 (?)	22.30	23.70	23.00	21.40	Trace
10	3.8	22.55	22.58	24.00	23.30	21.55	3.5
11	12.9	22.72	22.72	24.12	23.44	21.70	12.9
12	0.6	23.00	23.00	24.40	23.70	22.00	0.5
13	5.2	23.65	23.59	25.00	24.29	22.54	5.8
14	Trace	23.72 (?)	23.72 (?)	25.10 (?)	24.40 (?)	22.70 (?)	Trace
15	1.2	24.00	24.00	25.40	24.70	23.00	0.8
16	Trace	24.40 (?)	24.40	25.80	25.10	23.40	Trace
17	Trace	24.55 (?)	24.50	25.90	25.20	23.50	Trace
18	0.5	24.71	24.70	26.05	25.35	23.70	0.4

[a] GLC performed on a Loe Model 160 gas chromatograph equipped with H_2 ionization detector. Acetonides were examined on three different columns: a 1/8 in. × 1.5 ft stainless steel column packed with 3% JXR on silanized Gas Chrom. Q 100–200 mesh, 280 C, He at 60 ml/min for chain lengths up to C_{30}; a 1/8 in. × 8 ft stainless steel column packed with 8% EGSSX on silanized Gas Chrom. P, 100–200 mesh, He at 60 ml/min, 190 C; and a 1/8 in × 9 ft stainless steel column packed with 3% JXR on Gas Chrom. Q, 220 C, He at 60 ml/min. (Applied Sciences Inc. State College, Pa. supplied all phases and supports.) Aldehydes were examined on the EGSSX column at 170 C, 60 ml He/min.

[b] Acetonides were from fraction 15 Chromatogram I Figure 1. To establish the iso and ante-iso structures of the diol chain three fractions of acetonides were collected by preparative GLC procedures as described in (2): peak No. 1–5, peak No. 6 and peak No. 7–18. The acetonides were hydrolyzed with 6N H_2SO_4 for 14 hr, the diols extracted with ether, washed with water, and dried over KOH in a vacuum dessicator. The diols were then oxidized with $KMnO_4$ (12). Peak No. 6 gave only acetone which showed it to have the iso structure whereas collected peaks 1–5 and 7–18 gave both acetone and 2-butanone showing that both iso and anteiso structures were present.

[c] Carbon numbers, determined by the method of Woodford and Van Ghent, were measured as previously described (2). Entries followed by (?) were minor peaks whose carbon numbers were difficult to determine accurately.

[d] 1,2-Diol standards were prepared by $LiAlH_4$ reduction of C_{16}, C_{18}, C_{22} and C_{26} α-hydroxy fatty acids (Applied Sciences, Inc.).

[e] 2,3-*Cis* and 2-3-*trans* diol acetonide standards were from preen gland lipids of the hen as in (8). Homologues obtained were C_{22} through C_{25}.

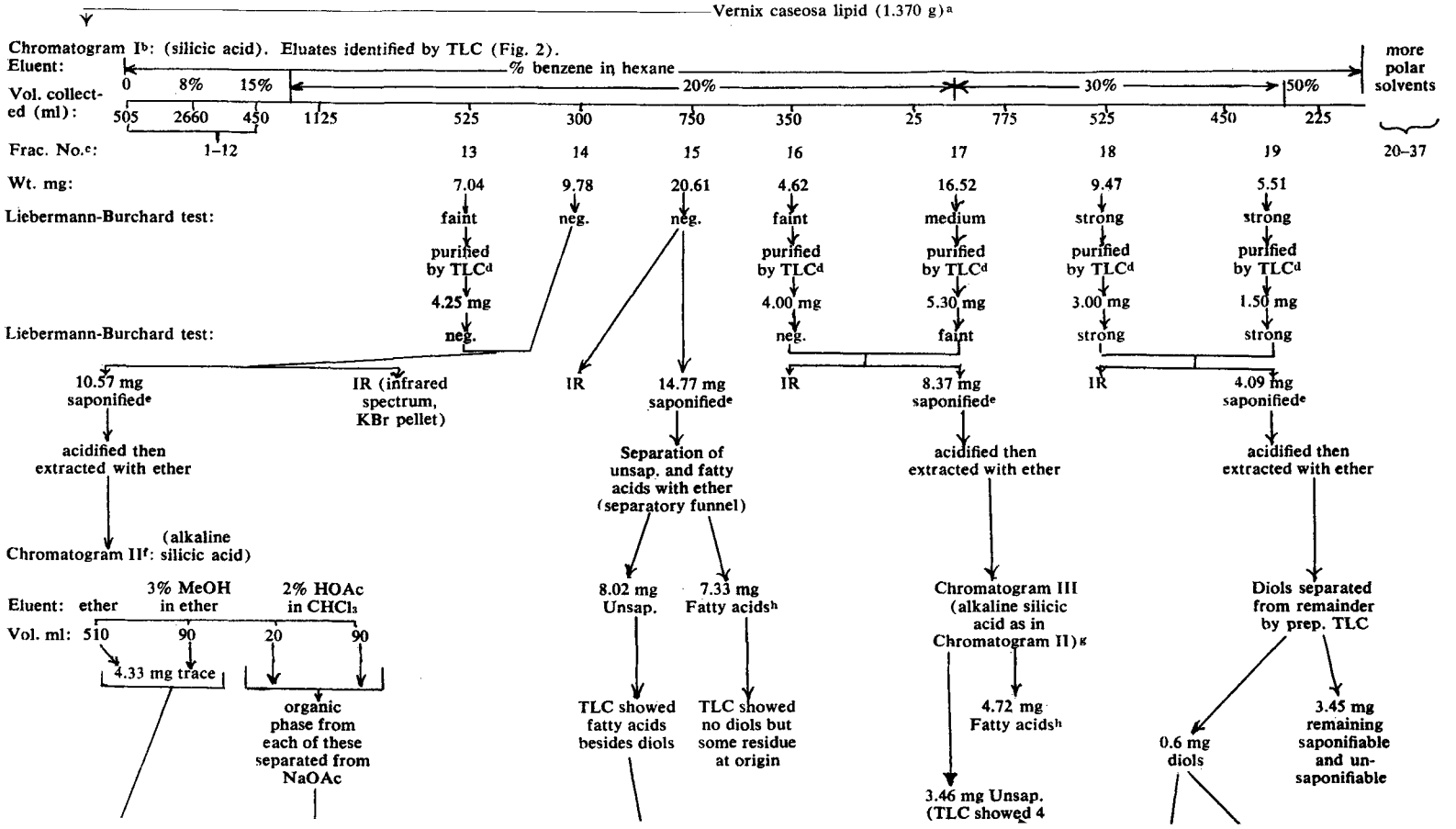
[f] Aldehydes obtained as in footnote 1 Figure 1. Aldehyde standards C_{17}, C_{21} and C_{25} were prepared similarly from C_{18}, C_{22} and C_{26} synthetic 1,2-diols, and C_{20} to C_{23} aldehydes were also obtained from the periodic acid oxidation of hen 2,3-diols (8).

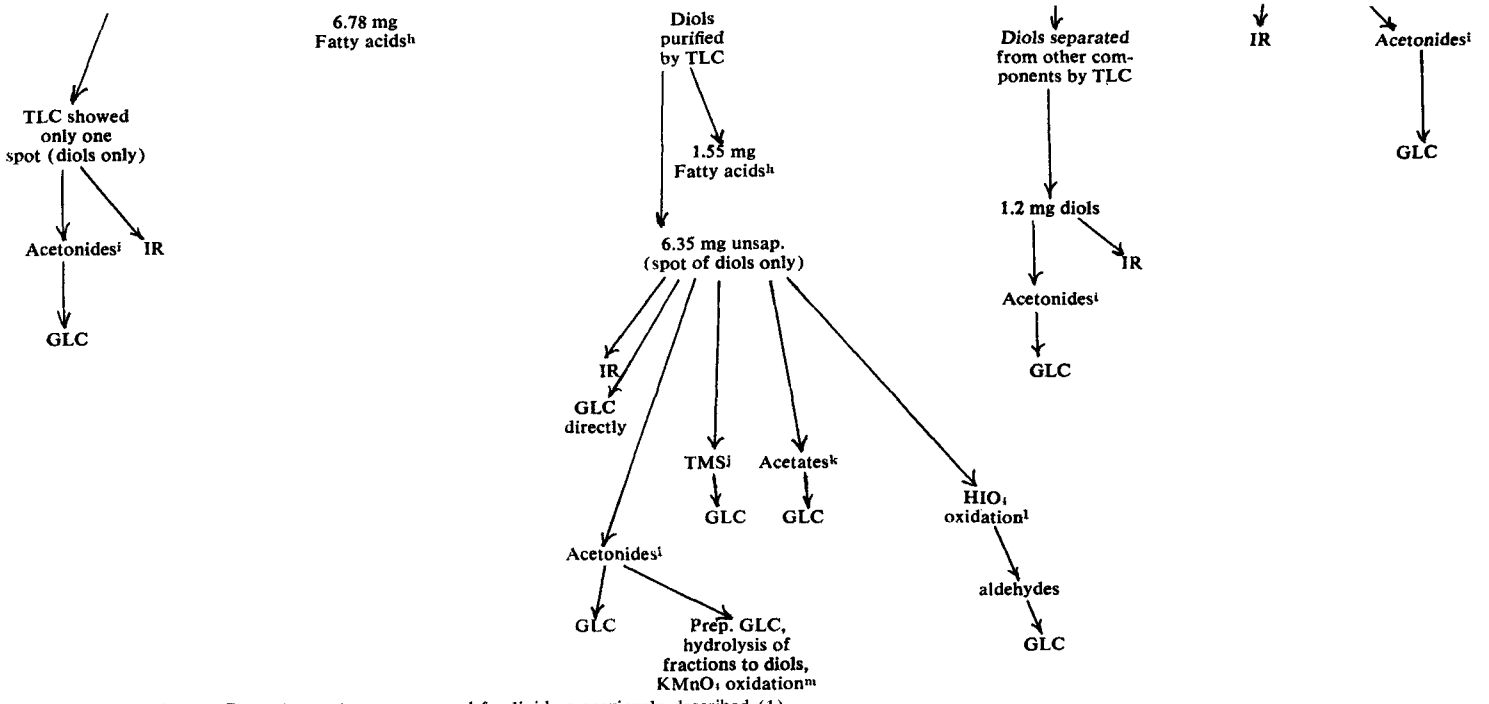
grating above the bulk of the fraction by preparative TLC (Fig. 2a and c). Apparently some very polar sterol esters had overlapped with early diester fractions. The saponification products of combined fractions 13 and 14 were separated quantitatively on alkaline silicic acid (chromatogram II Fig. 1). Again only 1,2-diols and fatty acids were recovered showing that these fractions, too, were diesters of 1,2-diols.

Later fractions (17 to 19) gave increasingly positive Liebermann-Burchard tests which persisted even after each fraction was purified to give one spot by TLC (Fig. 2c). Saponification products of fractions 18 and 19 showed by TLC in two systems, besides fatty acids and 1,2-diols, material migrating where α-hydroxy fatty acids, sterols and fatty alcohols migrated. Two additional types of diesters could account for these products: α-hydroxy fatty acids esterified on the OH group with an unsubstituted fatty acid and on the COOH group with either a sterol or a fatty alcohol. Kärkkäinen et al. (4) also found evidence for the latter type of diester. Of the three types of diesters apparently present in vernix caseosa, diesters of diols are in greatest abundance.

The alkane diols of fractions 13-19 formed acetonides, which on hydrogenation and GLC analysis on a polyester (EGSSX) column, showed no GLC pattern change from the original. Thus the alkane chains must have been saturated, in confirmation of earlier work (3,4). The free diols underwent periodic acid oxida-

FIG. 1. Isolation and analysis of diols from diesters of vernix caseosa.





[a] Vernix Caseosa from a Caucasian male was extracted for lipid as previously described (1).

[b] 110 g silicic acid (Unisil 100-200 mesh, Clarkson Chemical Co., Inc., Williamsport, Pa.) packed into a column 4.5 cm i.d. to 16.5 cm bed height.

[c] Only the work-up of fractions 13-19 (Fig. 2a,b) are discussed in this paper.

[d] TLC as in Figure 2 except 2 mg lipid were streaked on each plate, the plates developed, sprayed with water to make spots visible, diester region scraped, scrapings dried and lipid extracted with freshly redistilled ether.

[e] Diesters were saponified for 2 hr. with 10% KOH (wt/vol) in ethanol-water (9:1 v/v) under reflux in a N_2 atmosphere.

[f] Silicic acid (Unisil) made alkaline as in (9) and 2.5 g packed in a column 1 cm i.d. to a bed height of 8 cm.

[g] Same conditions as in f except 2% formic acid (as recommended in ref. 9) rather than 2% acetic acid was used to elute fatty acids. This avoided the necessity of removing a salt (e.g. NaOAc) with a water wash.

[h] Analysis of these fatty acids showed unusual double bond patterns to be reported subsequently.

[i] Acetonides prepared as in (8).

[j] TMS derivatives prepared as in (10).

[k] Acetates prepared as in (2); purity checked by TLC with hexane/ether (80/20) solvent.

[l] HIO_4 oxidation carried out essentially as in (12): 0.5 mg (—1.7 μmoles) diol treated in the dark with 0.034 ml 1M H_5IO_6 (3.4 μmoles) plus 0.2 ml redistilled tetrahydrofuran (J. T. Baker Chem. Co., Phillipsburg, Pa.) for 4 hr. at room temperature, then 2 ml water added and aldehydes extracted twice with 2 ml portions of hexane and the pooled extracts washed with water.

[m] See footnote b Table I.

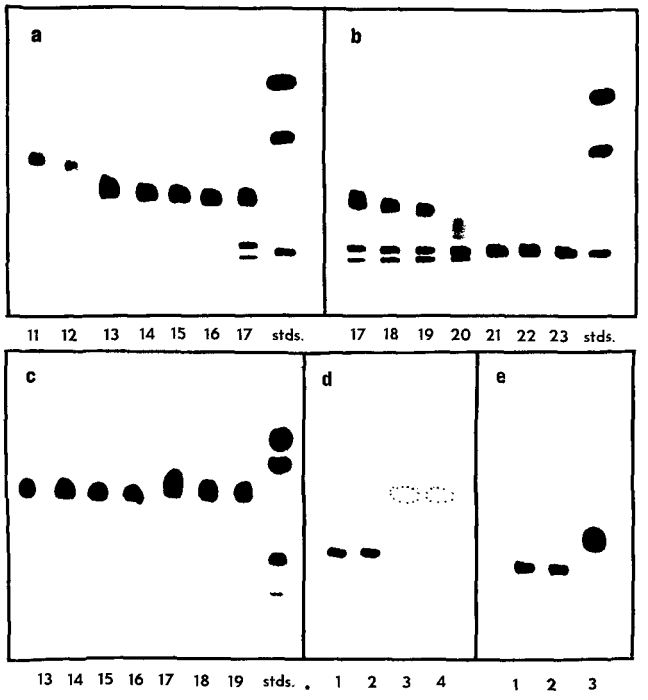


FIG. 2. a and b are photographs of TLC plates of fractions 11 through 23 of Chromatogram I (Fig. 1). TLC procedures as used by us (13) were those of Rouser et al. Sixty micrograms of each fraction, and standards consisting of 30 μg each of squalene, cholesteryl oleate and triolein were applied to each plate and the plate developed with hexane/ether (95/5 by volume). The "diester region" is the area between cholesteryl oleate (R_F = 0.5) and triolein (R_F = 0.1).

2c. Procedures as in 2a and b except development solvent was hexane-ether (92:8); 50 μg each of fractions 13 through 19, previously purified by preparative TLC where indicated in Figure 1 were applied to the plate. Standards were as in 2a and b.

2d. Procedures as in 2a and b except that the development solvent was $CHCL_3$-acetone -HOAc (80/20/1 by volume); 30 μg respectively applied in lanes (1) 1,2-hexacosane diol, (2) unsaponifiable of fraction 15, (3) saponifiable of fraction 15, (4) palmitic acid.

2e. Procedures as in 2a and b except that the solvent was ether, lanes (1) 40 μg unsaponifiable of fraction 15, (2) 40 μg of 1,2-eicosane diol, (3) 100 μg of 2,3-diols.

tion and GLC analysis of the resultant aldehydes showed material with carbon numbers corresponding to four homologous series (Table I): iso (71.7%) anteiso (19.1%) normal (8.7%) and branched chain of an unknown type (0.5%). That the chains were truly iso and anteiso was proved by $KMnO_4$ oxidation of free diols (obtained from hydrolysis of collected fractions of acetonides) which yielded acetone and 2-butanone respectively (Table I footnote b). Infrared spectra of the original diols showed a doublet at 1365 and 1380 cm^{-1} also consistent with the iso structure.

GLC retention data of the acetate, trimethyl silyl ether (TMS), and especially the acetonide derivatives of the diols support the TLC data that the positions of the OH groups are 1,2- rather than 2,3-. Table I shows that when the peaks of the diol acetonides of fraction 15 were plotted on three different standard curves (i.e. either 1,2-; 2,3- *cis;* or 2,3-*trans* diols), only the 1,2-diol standard curve gave retention data that matched exactly the aldehyde retention data predicted for periodic acid degradation of each chain by one C-atom. If 2,3-*cis* or 2,3-*trans* diols standard curves were used, and degradation of each chain by 2 C-atom assumed, the retention data of the acetonides did not match those of the aldehydes.

GLC of the diol acetonides from fractions 13 to 19 showed that the later fractions generally had shorter diol chain lengths (Table II). A calculated homologue distribution of all the 1,2-diols of these diesters fractions corresponded better to the data of Kärkkäinen et al. (4) (assuming that their homologues

TABLE II

Percentage Composition of Homologues of Alkane Diols from Chromatographic Fractions of Vernix Caseosa Diesters[a]

Peak numbers	1,2-Diol carbon numbers[b]	Fraction numbers of chromatogram I Figure 1				Calculated composition of total diols[c]
		13 and 14	15	16 and 17	18 and 19	
		%	%	%	%	%
1	19.65	9.8	11.6	15.5	39.6	12.4
2	20.00	1.5	1.9	3.1	4.0	2.0
3	20.65	2.1	3.1	2.8	2.5	2.7
4	20.75	4.3	6.4	5.0	6.8	5.6
5	21.00	0.9	1.1	3.5	1.7	1.3
6	21.65	48.4	47.5	44.0	23.2	46.5
7	21.72	trace	trace	trace	trace	trace
8	22.00	3.8	3.7	3.5	2.7	3.7
9	22.40	trace	trace	trace	trace	trace
10	22.55	3.8	3.8	3.9	2.2	3.7
11	22.72	14.1	12.9	10.8	7.0	12.9
12	23.00	0.8	0.6	0.9	0.5	0.7
13	23.65	8.5	5.2	4.7	6.8	6.4
14	23.72	trace	trace	trace	trace	trace
15	24.00	0.7	1.2	1.1	1.2	1.0
16	24.40	trace	trace	trace	trace	trace
17	24.55	trace	trace	trace	trace	trace
18	24.72	0.8	0.5	0.5	0.6	0.6

[a] Area percent calculated with assistance of Dupont 310 Curve Resolver.

[b] As acetonides (Table I). GLC performed on 1/8 in. × 9 ft column packed with 3% JXR programmed from 218 C to 260 C at 2°/min, He flow at 60 ml/min.

[c] Calculation based on weight recovery of diols for fractions 13 to 19.

were iso and anteiso instead of straight as they reported) rather than to the data of Downing (3).

HWEI C. FU and N. NICOLAIDES
University of Southern California
School of Medicine
Section of Dermatology
Los Angeles, California 90033

ACKNOWLEDGMENT

This work was supported by research grant AM-10010 from the National Institute of Arthritis and Metabolic Diseases, U.S. Public Health Service and by the Birely Foundation, Los Angeles, California.

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[Received September 20, 1968]

LETTER TO THE EDITOR

Amaurotic Idiocy

Sir: A general term such as amaurotic idiocy which was used originally by Sachs to designate a specific disorder (now known as Tay-Sachs disease) with blindness (amaurosis) and extreme mental deficiency (idiocy) may lose much of its significance as cases are encountered that have both of these major features but are otherwise different. Zeman (Lipids *4,* 76-77, 1969) has pointed out that the situation has become complex and confusing and that serious consideration must be given to abandonment of the term amaurotic idiocy in the classification of disease.

If blindness, severe mental deficiency, and evidence of abnormal intraneuronal lipid accumulation are accepted as defining amaurotic idiocy, the following subdivisions can be recognized:

I. Gangliosidoses.
   A. GM_2 gangliosidosis (Tay-Sachs disease).
   B. GM_2 gangliosidosis arising from hexosaminidase deficiency (Sandhoff et al., Life Sci. *7,* 283-288, 1968).
   C. GM_1 gangliosidosis, Type I (Derry et al., Neurology *18,* 340, 1968) reported to be a β-galactosidase deficiency (Okada and O'Brien, Science *160,* 1002-1004, 1968).
   D. GM_1 gangliosidosis, Type II (Derry et al., loc. cit.).

II. Batten's Syndrome.

Pigment deposition without evidence of abnormal sphingolipid metabolism including cases of the late infantile (Bielschowsky-Jansky), juvenile (Spielmeyer-Vogt), and adult (Kufs) types.

It is to be noted that IB rests upon one recent report only and that there are differences of opinion regarding IC. The deposition of lipofuscin (ceroid?) in II, while a consistent and prominent feature, is known to occur in other conditions and thus should not be considered as a firm basis for special classification. The terms "lipofuscin" and "ceroid" are frequently used interchangeably for insoluble deposits believed to be formed, at least in part, from lipid. Little is known, however, of the source, composition, and mode of formation of such deposits. The chemical characterization of lipofuscin (ceroid) isolated free of other cellular structures is an important area for future research in lipid chemistry. Lipofuscin (ceroid) deposition is found in various pathological states, can be induced by various experimental means, and seems always to involve lipid, most of which is originally present in the cell surface membrane, the endoplasmic reticulum, mitochondria, nuclei, etc. These features suggest that such deposits may appear following damage of cell membranes. It is possible that in hereditary diseases the mutation may involve membrane protein when lipofuscin deposits are found in the absence of other lipid changes. This possibility can be explored by analysis of pure subcellular particulate preparations.

It is apparent that disorders with abnormal amounts of gangliosides can be classified adequately without reference to amaurotic idiocy. While retention of the amaurotic idiocy designation for cases Zeman refers to as Batten's disease (syndrome) can be justified on the basis of frequent use in the literature, the apparently erroneous assumption that the amaurotic idiocies are all disorders of ganglioside metabolism does provide justification for abandonment of the term entirely. There is a well established trend away from reporting of individual cases as a type of amaurotic idiocy. Designations such as systemic late infantile lipidosis, gangliosidosis, myoclonic variant of cerebral lipidosis, Batten's disease, etc. having been preferred by various groups. When nearly all literature reports are found to use designations other than amaurotic idiocy, justification for continued use in the literature will be removed. Classification problems will be fully clarified as specific defects are discovered.

GEORGE ROUSER
Section of Lipid Research
Division of Neurosciences
City of Hope Medical Center
Duarte, California
RONALD R. WADE
Department of Neurology
Presbyterian-St. Luke's Hospital
Unversity of Illinois College of Medicine
Chicago, Illinois

[Received April 7, 1967]

Acid Lipase of the Castor Bean[1]

ROBERT L. ORY, Southern Regional Research Laboratory,[2] New Orleans, Louisiana 70119

ABSTRACT

The acid lipase of the castor bean is present in the dormant seed. It is extracted from the fat pad obtained by centrifuging a macerate of the seed in pH 7.0 buffer containing cysteine and ethylene diaminetetraacetic acid. The pH optimum of the enzyme is 4.2; it is rather heat-stable, and is inhibited by mercurials and sulfhydryl reagents. Maximum hydrolysis of saturated triglycerides occurs with fatty acids of chain length C_4 to C_8; unsaturated C_{18} triglycerides are hydrolyzed at a slightly lower rate.

This lipase is a three-component system consisting of the apoenzyme, a lipid cofactor (a cyclic tetramer of ricinoleic acid), and a protein activator (a small, heat-stable glycoprotein which appears to be related to some of the castor allergens). Maximum lipolysis requires all three components. Lipase activity is associated with the spherosomes, the subcellular site of oil storage in the endosperm.

INTRODUCTION

The castor bean contains two lipases. One is present in resting seed and has an acid pH optimum (1-3); the other appears after several days of germination and has a pH optimum near neutral (4). This paper describes work on the dormant seed enzyme, or acid lipase.

PREPARATION AND PROPERTIES

The procedure presently employed to prepare the enzyme is shown in Figure 1.

The dehulled castor beans were ground several times by mortar and pestle in 0.1 M phosphate buffer, pH 7.8, containing 0.05 M cysteine and 0.01 M ethylenediaminetetraacetic acid, the macerate filtered through cheesecloth, and then centrifuged at high speed to yield a fatty layer, an aqueous supernatant, and the precipitated debris (5). This step serves two purposes. First, ricin and various allergenic proteins known to be in the castor bean are removed in the aqueous phase and the debris which are discarded, and the lipase is concentrated in the fatty layer obtained after centrifugation. This fat pad was extracted four times with ether and saturated salt solution to remove all of the neutral lipids and some inactive protein, leaving a particulate material which was dialyzed against water and freeze-dried to yield the crude lipase preparation. This lipase preparation is apparently a complete system which hydrolyzes most glyceride substrates very rapidly, according to first-order kinetics. It requires neither fatty acid acceptors such as calcium or albumin, nor added emulsifiers and is quite stable if kept dry in sealed containers (1). To start the reaction, simply add enzyme, substrate and water; then lower to pH 4.2 with acetic acid. Activity is measured by titrating the fatty acids released by the enzyme in 10 min with 0.1 N sodium hydroxide.

In Figure 2 is shown the pH activity curve of the enzyme at room temperature. The pH optimum is about 4.2 to 4.5. There is a very sharp falling off in activity, especially on the higher side. The cessation of activity above pH 5.5 was an important property in later attempts to localize the enzyme at the subcellular level.

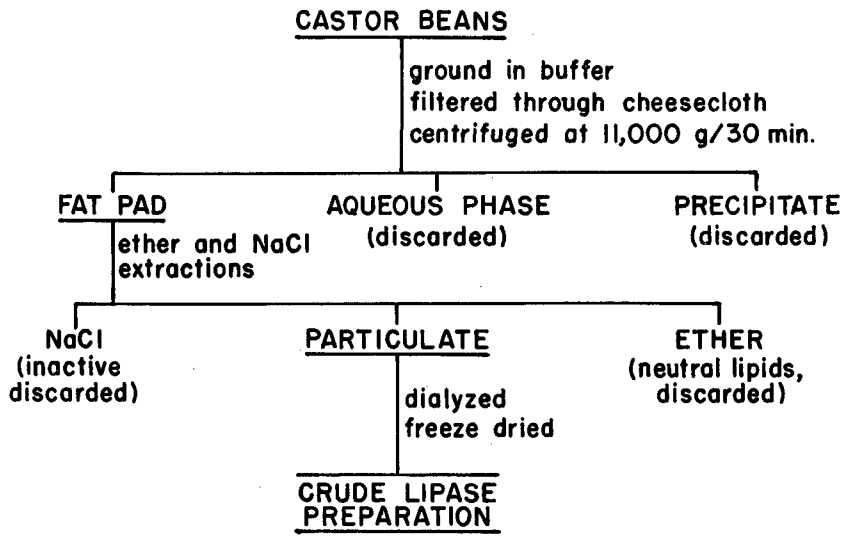
In Figure 3 the effect of heat on the enzyme for various periods of time is shown. The enzyme is fairly stable at 60 C. At 45 C (not shown here) it is stable for much longer periods of time. This property was also utilized in the attempts to determine the subcellular site of the enzyme.

As mentioned earlier, cysteine was added to the initial buffer extracts. This lipase is a sulfhydryl enzyme. It was completely inhibited by mercuric ion and organomercurials, such as parachloromercuribenzoic acid, but the inhibition was reversed by adding excess cysteine before testing. While mercury at low concentrations was a potent inhibitor, lead ion, another heavy metal, was ineffective at the usual concentrations (1) and was utilized in the histochemical localization of the enzyme.

Investigations on the effect of fatty acid chain length of triglycerides on activity of the lipase showed that the enzyme was completely inactive on triacetin at all concen-

[1] Presented at the AOCS-AACC Joint Meeting, Washington, D.C., April 1968.

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



CASTOR BEAN LIPASE PREPARATION

FIG. 1. Schematic diagram for preparation of the acid lipase of the castor bean.

trations studied (2). The castor lipase showed a preference for triglycerides of C-8 chain length. No experiments were conducted on saturated triglycerides of chain lengths longer than C-12 because at this point a solubility problem developed. The C-18 unsaturated fatty acid triglycerides: cottonseed oil, trilinolein, triolein and triricinolein, were hydrolyzed about 40-50% slower than the C-8 saturated triglyceride (2). Relative rates of activity of the lipase on the monohydric alcohol esters, butyl ricinoleate and methyl ricinoleate, showed that the enzyme was relatively inactive on these. This suggests that the castor lipase may be considered a true lipase rather than an esterase. Falk and Sigiura (6) reported the separation of an esterase from castor beans by salt extraction. The sodium chloride extraction used in the preparation of this lipase (Fig. 1) would have removed this esterase.

SEPARATION OF TWO COFACTORS

While attempting to solubilize the enzyme by the *n*-butanol procedure of the late Morton (7), the first of the two cofactors was separated. Aqueous butanol produced no changes but if dry butanol were used, an oily material was removed from the particulate material and the protein residue showed drastically reduced lipase activity. The butanol-extracted material had no lipolytic activity *per se*, but when this was added back to the extracted apoenzyme, activity was completely restored (8).

To identify this material, six different analytical techniques were employed to determine its structure: elemental analysis, molecular

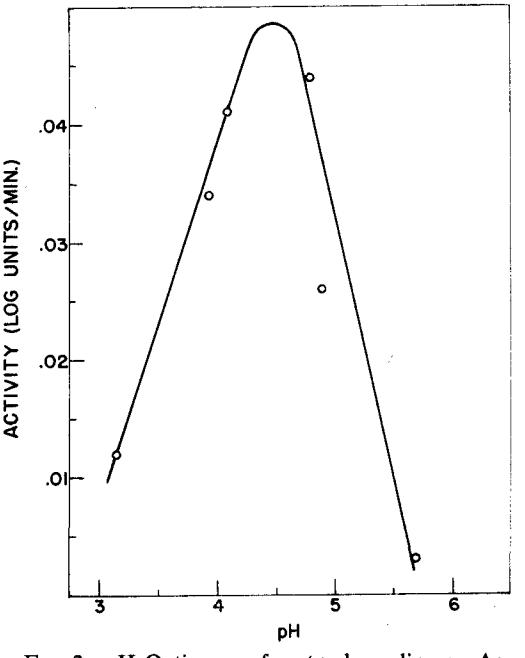


FIG. 2. pH Optimum of castor bean lipase. Activity is measured as the decrease in substrate concentration/min/mg. enzyme.

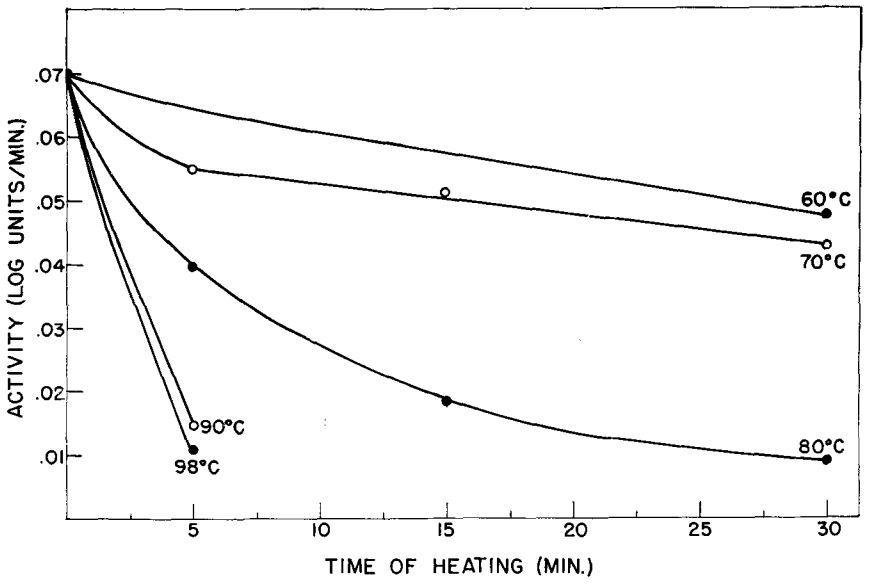
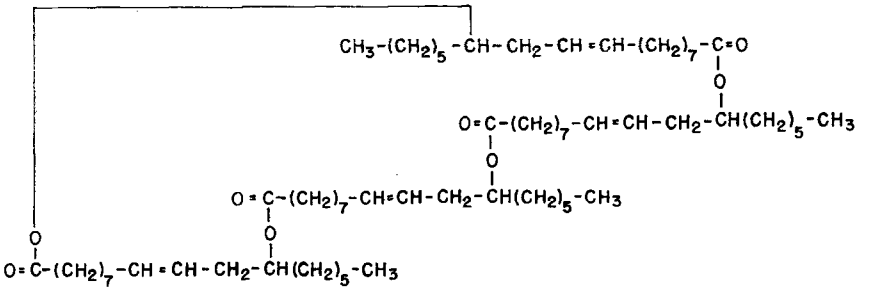


FIG. 3. Effect of heat on activity of castor bean lipase. Enzyme was heated in a water bath at designated temperatures for times indicated, cooled, and tested as in Figure 2.

weight by osmometry; comparison on glass paper and thin layer chromatography to various lipids and ricinoleate derivatives; infrared, ultraviolet, and nuclear magnetic resonance spectroscopy; and finally, chemical degradation and comparison to authentic ricinoleic acid degradation products. From these results (9), the structure of this lipid cofactor was shown to be a cyclic tetramer of ricinoleic acid. This is shown in Figure 4. Infrared spectra showed many similarities to ricinoleic acid, but there were some discrepancies. The NMR spectra likewise showed similarities to that of ricinoleic acid with one reservation, the absence of free hydroxyl or carboxyl groups. This finding, along with the extremely large molecular weight, confirmed the cyclic structure shown. How this is bound to the enzyme in such a way that the initial ether extractions failed to remove it is not known. However, it appears to be some sort of stable linkage since only dry *n*-butanol removed it.

Though the exact role of the lipid cofactor is unknown, one function may be that of a natural emulsifier. Since added emulsifiers were not required for maximum activity, the presence of a natural emulsifier was suggested. Experiments undertaken to determine the nature of the particles before and during active lipolysis showed that the lipid cofactor promotes emulsification of enzyme and substrate whether or not the enzyme's lipolytic function was inhibited (10).

During lipolysis tests, the turbid reaction



LIPID COFACTOR OF CASTOR BEAN LIPASE

FIG. 4. Lipid cofactor for castor bean lipase.

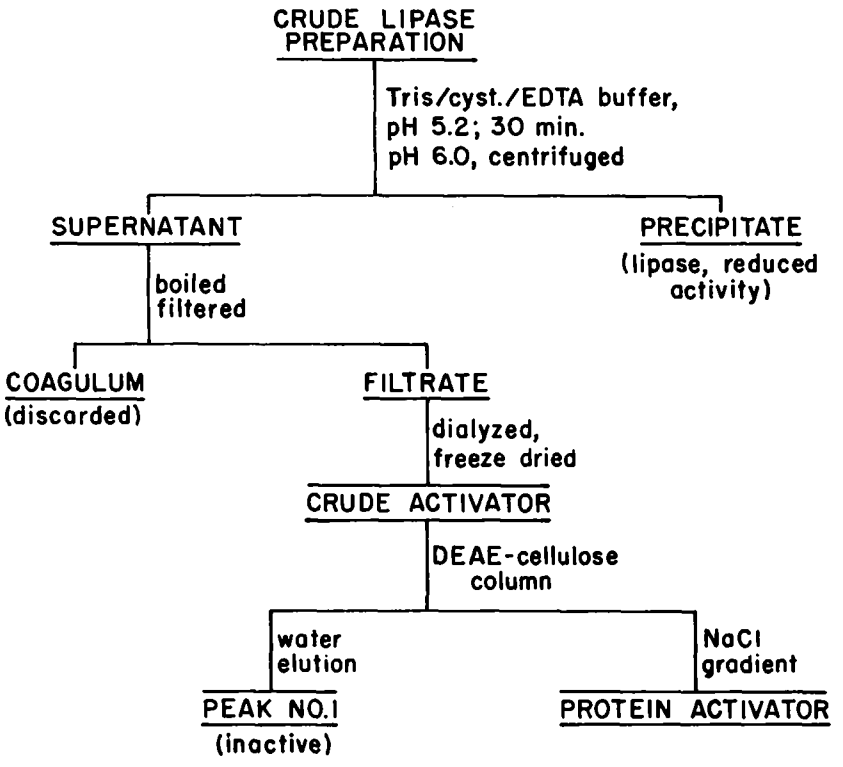


FIG. 5. Schematic diagram for separation of protein activator from the castor bean lipase.

mixture at pH 4.2 appeared to clarify somewhat when titrated to pH 8.5 at the end of a reaction, suggesting solubilization of some protein. This led to the separation of the second factor from the enzyme shown graphically in Figure 5.

If the crude lipase preparation were incubated at pH 5.2 for 30 min, the pH raised to 6 and centrifuged, a very clear supernatant with no lipase activity and a particulate apoenzyme with greatly reduced activity was obtained (11). When this supernatant material was added back to the extracted lipase, activity was completely restored. This material was boiled, the coagulum filtered off, the filtrate was dialyzed and freeze-dried. The white product was fractionated on a DEAE- cellulose column to yield two components; the first eluted by water, the second by means of a salt gradient. Adding back the crude activator, the water-eluted peak and the salt-eluted peak material to the extracted apoenzyme (the enzyme from which this protein activator is removed) showed that the entire amount of the activating principle was in the salt-eluted fraction from the DEAE-cellulose column (11).

This small heat-stable protein appeared to be similar or related to castor bean allergens. Crude allergens were prepared by extracting the castor beans with tris and phosphate buffers and the CB-1A classical allergen was prepared by the method of Spies and Coulson (12). The CB-1A allergen is essentially a water extract of castor beans with a series of alcohol precipitations and a lead acetate precipitation step. It was found to be a heterogenous material showing 7 or 8 bands upon gel electrophoresis. The buffer-extracted proteins were boiled, filtered, dialyzed and freeze-dried in the same manner as the protein activator.

These allergen fractions were compared to the protein activator. By gel electrophoresis, all appeared to have common bands. In lipolysis tests, both of the buffer extracted allergens increased lipase activity of the extracted apoenzyme but the CB-1A allergen did not. However, in one step of the procedure for preparing CB-1A there is a lead acetate precipitation step which, according to the authors, removes certain allergens and antigens from the CB-1A preparation (13). This lead-precipitated component was partially purified by re-

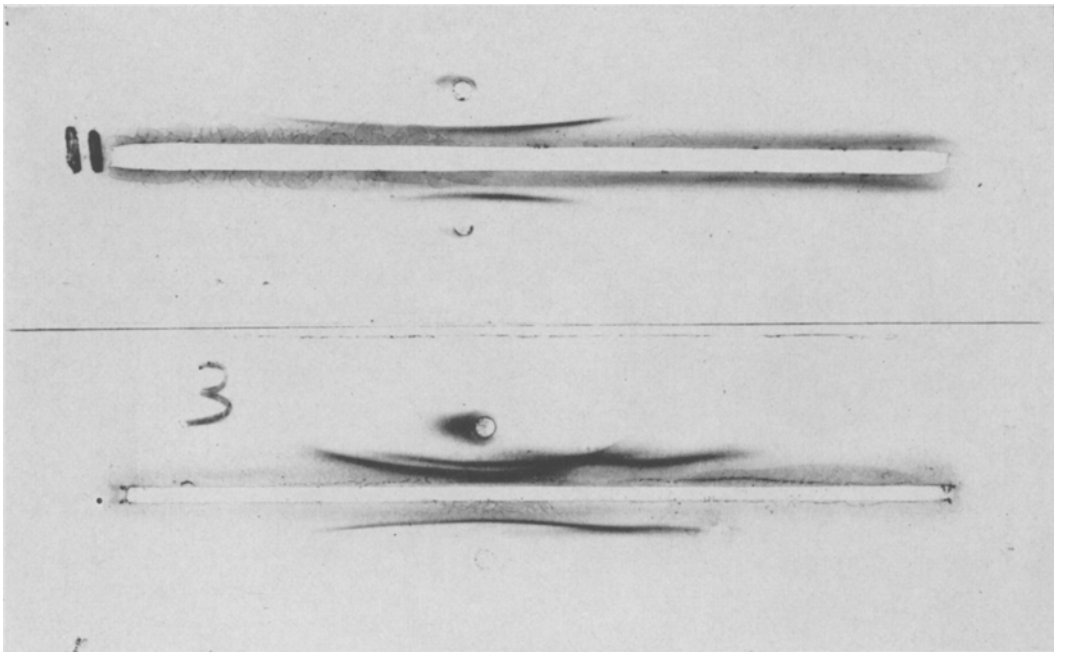


FIG. 6. Comparison of the protein activator and the lead-precipitated antigens from the CB-1A allergen by immunoelectrophoretic analysis. Proteins were separated first by electrophoresis, 2 hr at 200 v in 1% Ionagar, pH 8.2 Veronal buffer; antigens and antisera described in text.

moving the lead with H_2S, dialysis, and freeze-drying. This purified lead-precipitated fraction greatly increased activity of the extracted apoenzyme, suggesting a possible role for allergens in lipid metabolism of the seed. This material compared quite favorably to the activator by gel electrophoresis, amino acid analysis, ultracentrifuge and biological activity. A more precise means of comparing the two was sought and the immunoelectrophoretic analysis technique of Grabar and Williams (14) was employed. Immune sera containing antibodies to castor proteins were used to compare the purified protein activator to the lead-precipitated fraction from the CB-1A allergen. The results are shown in Figure 6. The upper wells of each of the microscope slides (11 and 3) contain a mixture of the two antigens. For the lower wells, the lower slide contains the lead-precipitated component and the upper slide contains the activator. The central canals contain immune serum as follows: in slide 3, antibodies to the total extracted castor bean proteins and in slide 11, antibodies to the CB-1A allergen preparation. As can be seen, the electrophoretic mobilities of both materials are quite similar. However, the shapes of the two precipitin arcs are different; that of the activator being a sharp arc while the precipitin band for the lead-precipitated fraction is an elongated, rather flat arc. That the two materials are different is shown in the upper well of slide 3 where the precipitin arcs cross through each other rather than forming one continuous band. Based on these and other similar tests on various allergens, it must be concluded that the activator is not one of the well characterized allergens. However, it is interesting that a fraction from the characterized allergens can function in lipid metabolism of the seed.

The role of this protein activator is still not known but it is distinct from the lipid cofactor. The lipase was completely extracted for both cofactors, then the effects on lipolysis of adding back each of the cofactors separately and together was measured. The results showed that both are required for complete restoration of activity of the lipase (11). Thus, the castor bean acid lipase appears to be at least a three-component system in the seed.

SUBCELLULAR LOCALIZATION OF THE LIPASE

Since the lipase was always found in the fat pad after centrifuging a homogenate, it seemed that this fat pad might be a concen-

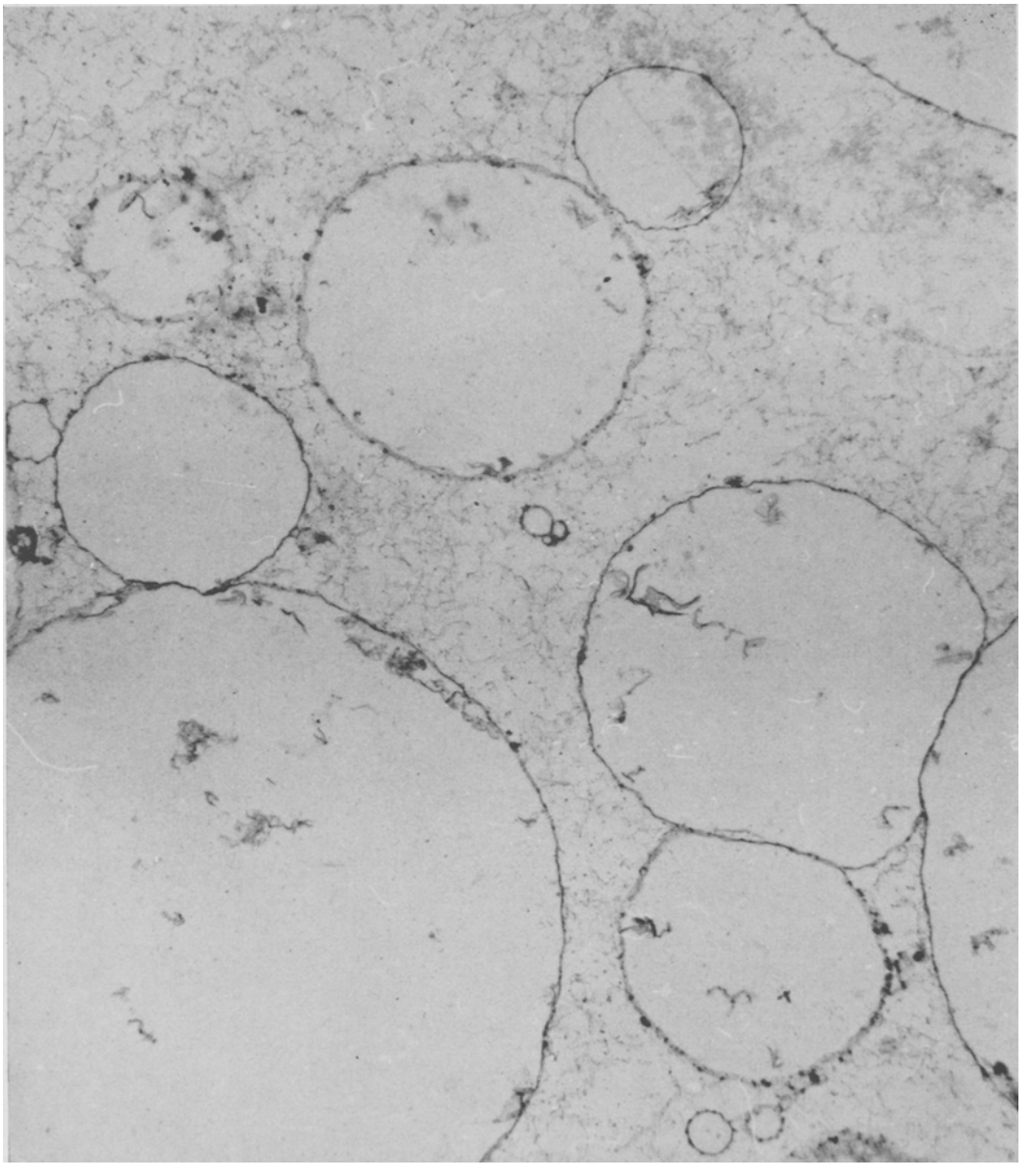


FIG. 7. Electron photomicrograph of castor bean spherosomes from fat pad. Castor oil has been extracted with hexane prior to fixing in glutaraldehyde, acetone dehydration and osmium staining; magnification about 8,000 (reduced approximately 30%).

trated form of spherosomes, the oil-containing organelles of the seed. Experiments were designed to test this hypothesis and to determine if the lipase was associated with the spherosomes or if it was floating about somehow with the cytoplasmic proteins surrounding the spherosomes.

The fatty layer was prepared as usual and at the same time a centrifuge tube containing a 4% agar solution was heated at 100 C to melt it, then cooled back to 45 C; agar is still liquid at this temperature. The fatty layer after extraction was thoroughly washed in water to remove cysteine and any interfering salts, placed in a sealed flask under nitrogen and equilibrated at 45 C, the same as the

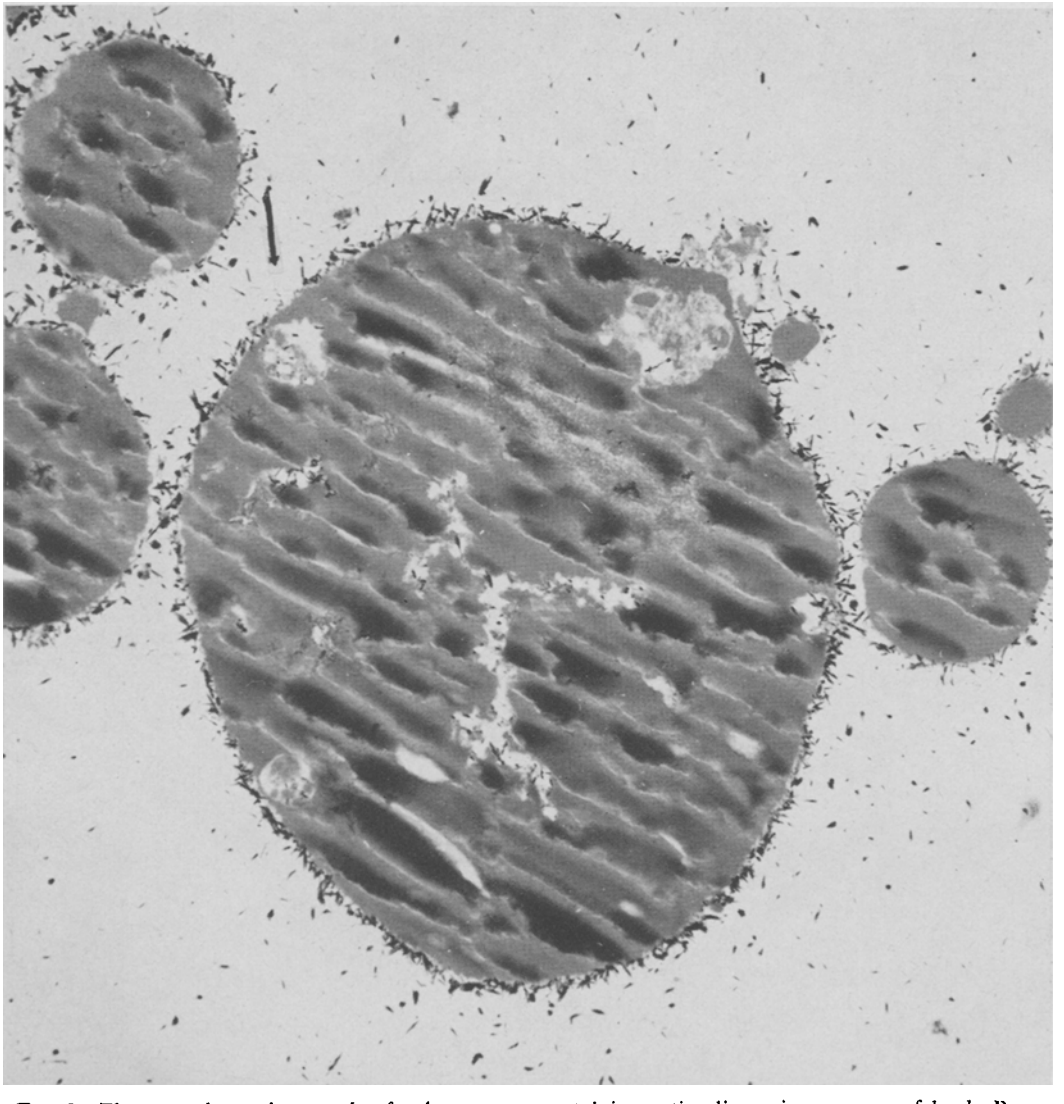


FIG. 8. Electron photomicrograph of spherosomes containing active lipase in presence of lead. Reaction conditions: 8 min at pH 4.2 in tris buffer containing 10^{-3} M lead acetate; lipolysis stopped by transferring tissues to pH 5.5; tissues washed in water, fixed in osmium, magnification about 10,000 (reduced approximately 30%).

agar. The enzyme is quite stable at 45 C. After this, the two were poured into a tube and immediately centrifuged for 5 min to form a solid mass at room temperature. This agar-fatty layer gel could be sliced into pieces 1 mm^3 in size for the various tests and for electron-microscopic examination. Some of the pieces were fixed in glutaraldehyde to immobilize the protein. The tissues were then extracted with hexane to remove all neutral lipids, counterstained with uranyl acetate and osmium and examined for the presence of spherosomes. In Figure 7 an electron photomicrograph of this tissue showing intact spherosome membranes is shown. Since the oil was removed by the hexane and acetone dehydration steps prior to osmium-fixation, the interior spaces appear vacant. However, the results do show that the fat pad contains the concentrated spherosomes (15).

The location of the lipase was determined by combining biochemical and histochemical methods. As mentioned earlier, while mercury completely inhibited the lipase, lead at nor-

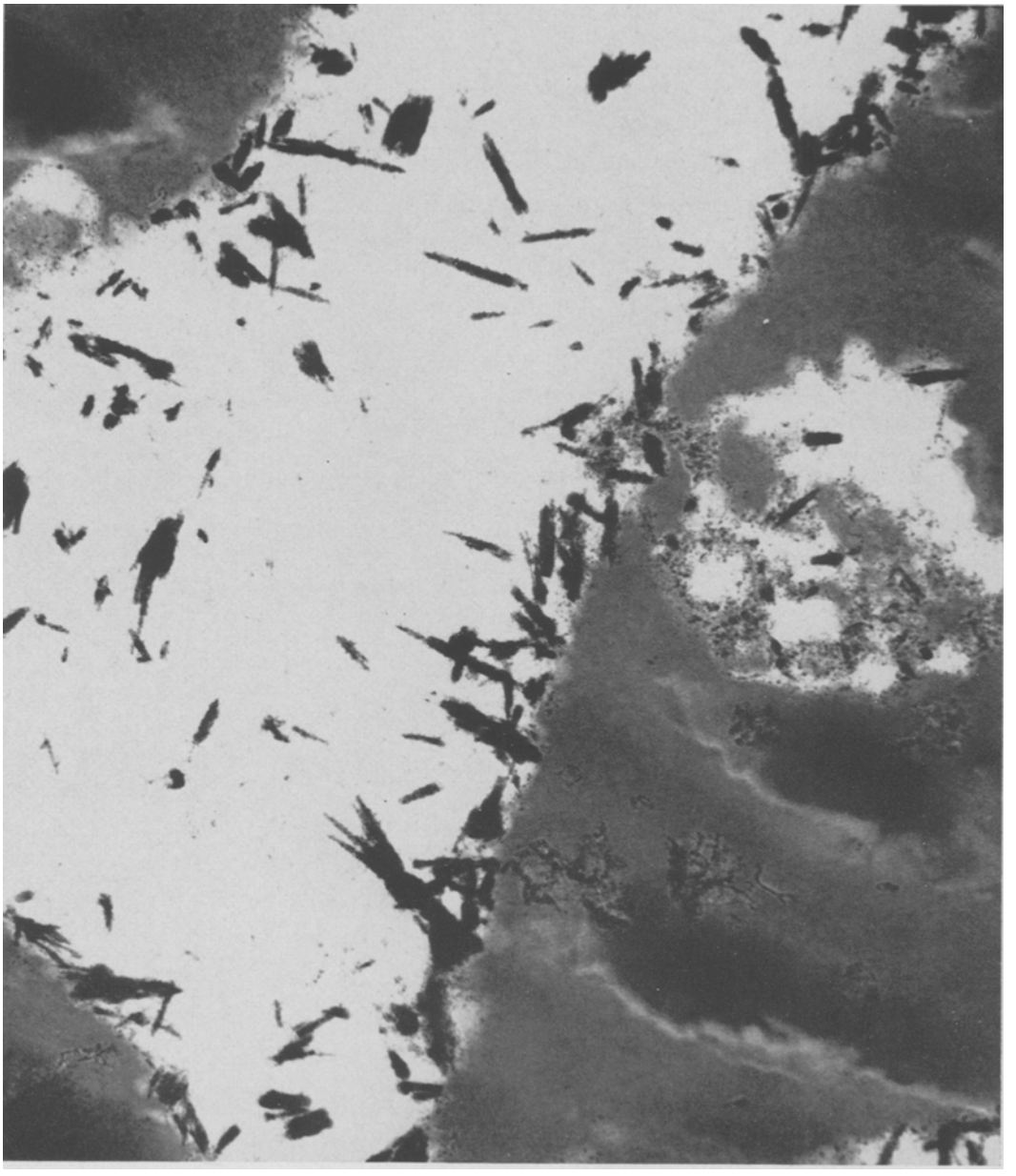


FIG. 9. Electron photomicrograph of spherosome containing active lipase in presence of lead. Same conditions as in Figure 8; magnification about 49,000 (reduced approximately 30%).

mal concentrations did not. Since lead is an electron-dense material, this was utilized to localize activity. Two types of controls were run along with the experimental pieces of agar-fatty layer gel as follows: some were controls at pH 7 without lead; some were controls at pH 5.5 with 10^{-5} *M* Pb^{++} but no lipolysis; the other tissues for assay of lipolysis in presence of lead were placed in pH 4.5 acetate buffer with 10^{-5} *M* Pb^{++} for different periods of time. Lead being a noninhibitor could be present without affecting lipase activity. But at each point where a fatty acid was released by the lipase, it should immediately bind to the lead to form an insoluble lead soap. As these soaps accumulated, electron-dense particles should appear at the point of hydrolysis.

Since both types of controls showed none

of the lead precipitates and were closer in appearance to the spherosomes in Figure 7, only the photographs of active tissue are presented. Figure 8 shows the results obtained with pieces which had been incubated for 8 min at pH 4.2 in presence of lead ions. At the end of the reaction period, the pieces of tissue were immediately transferred to a second vessel of pH 5.5 containing no lead. This stopped lipolytic activity and permitted unreacted lead ions to diffuse out of the pieces. The pH 7 controls with no lead were placed directly in water. After 15 to 20 min at pH 5.5, all pieces were transferred to water for 1 hr to completely remove all soluble unreacted lead ions. It is readily obvious that the lead fatty soaps are precipitated and concentrated around the spherosomes. As hydrolysis occurs, it is possible for the membranes to break or separate so that hydrolysis might take place within the membrane. The arrow in this photograph points to an area which is magnified in Figure 9. This shows that the membranes can break during active lipolysis but the concentration of lead soaps appears to be greater on the outside of the membranes rather than within.

From this it was concluded that the acid lipase of the castor bean is somehow associated with the spherosomes and acts either at, on, or as part of the spherosome membranes (15). In this respect, the spherosome might be likened to the casein micelles containing the milk lipase described earlier by Harper (16) and to the animal lysosomes described by deDuve (17). Lysosomes have been referred to as subcellular particles which contain the degradative enzymes, one being a lipase. Lysosomes have not been found in plants, though Yatsu and Jacks (18) have reported lysosomal-like acid proteinase and acid phosphatase activity in aleurone grains of cottonseeds.

ACKNOWLEDGMENTS

G. I. Pittman made the drawings, Jack Bergquist the photographs, and J. L. Daussant the immunoelectrophoretic analyses.

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[Received June 27, 1968]

Serum Lipoproteins: A Paper Electrophoresis Method Without Albumin in the Buffer

MOHAMMED MOINUDDIN and LINNARD TAYLOR
Lipid Standardization Laboratory, National Communicable Disease Center,
U.S. Department of Health, Education and Welfare, Atlanta, Georgia 30333

ABSTRACT

A paper electrophoresis method is described in which four serum lipoprotein components are separated without the use of albumin in the buffer. Tris-EDTA-boric acid buffer, Oil Red O staining solution prepared in 52.8% instead of the usual 60% alcohol, and solvent-extracted paper strips are the distinguishing features of this procedure. The system was effective not only in separating chylomicron, β- and α-lipoproteins into well-defined bands, but also in separating very low density or pre-β-lipoprotein distinctly in some samples and partially in others. The advantages of this procedure are low background staining and, in comparison to the procedure using albumin in the buffer, a sharper alpha band. This sharpened α-band gives a sharper peak in densitometric scanning. Electrophoresis could be performed for 16 hr or 4 hr. The 4-hr electrophoresis run produced electrophoregrams with even denser and sharper bands than the 16-hr run.

INTRODUCTION

The Jencks and Durrum (1) method for the separation of serum lipoproteins used in many laboratories, employs barbital buffer for electrophoresis and Oil Red O for staining. The system was efficient in resolving only three fractions, namely, the chylomicron, β- and α-lipoproteins of the serum. However, in 1963 Lees and Hatch (2) introduced the use of 1% albumin in barbital buffer and showed that the presence of albumin "provides sharper delineation of major lipoprotein components and a partial resolution of the very low-density lipoproteins."

Although the presence of albumin did help in the resolution of four lipoprotein bands, pre-β included, the α-lipoprotein band still was not very sharp. On the other hand, Sonnino and Gazzaniga (3) had previously shown that the Tris-EDTA-boric acid (TEB) buffer introduced by Aronsson and Gronwall (4) produced a sharper and denser α-lipoprotein band than did the barbital buffer.

In view of these observations, it seemed worthwhile to undertake a study to determine whether further improvement could be made in the resolution of lipoproteins in paper electrophoresis and, in addition, to find conditions under which albumin would not be needed. During the course of this study, a method was developed that permitted the separation of serum lipoproteins into four fractions without the addition of albumin to the buffer, and, at the same time, produced a compact and sharp α-lipoprotein band.

MATERIALS AND METHODS

Solvent-extracted Paper Strips

Whatman 3 MM paper strips were boiled in methanol for ½ to 1 hr and then left to soak overnight. Next, they were extracted in a modified Soxhlet extractor (Scientific Glass Apparatus Co.) with chloroform-methanol (2:1 v/v). The capacity of the extractor was 2200 ml and 250 strips were extracted at a time. The extraction was carried out for 10-12 complete cycles, requiring about 20 hr. The Soxhlet extraction process was repeated with diethyl ether. All solvents were reagent grade.

Preparation of Oil Red O Stain

The Oil Red O used in this study was manufactured by Chroma-Gesellschaft and distributed by Roboz Surgical Instruments, Washington, D.C. The procedure is the same as that reported by Jencks and Durrum (1) except that the alcohol solution used is 52.8% instead of 60%. The aqueous alcohol is prepared by mixing 95% alcohol (U.S.P.) and distilled water in the ratio of 5:4 (v/v).

Electrophoresis Procedure

Electrophoresis is carried out using the solvent-extracted strips in a Durrum-type cell (Beckman Model R) at room temperature or in a room held at 70 ± 2 F. TEB buffer (Tris 60.5, EDTA 6.0, and boric acid 4.6 g/l) with a pH of 8.9, is used as described by Aronsson and Gronwall (4). The electrophoresis time was either 16 hr using 5 ma constant current or 4 hr using 230 v constant voltage. Except for two minor modifications, all the steps in

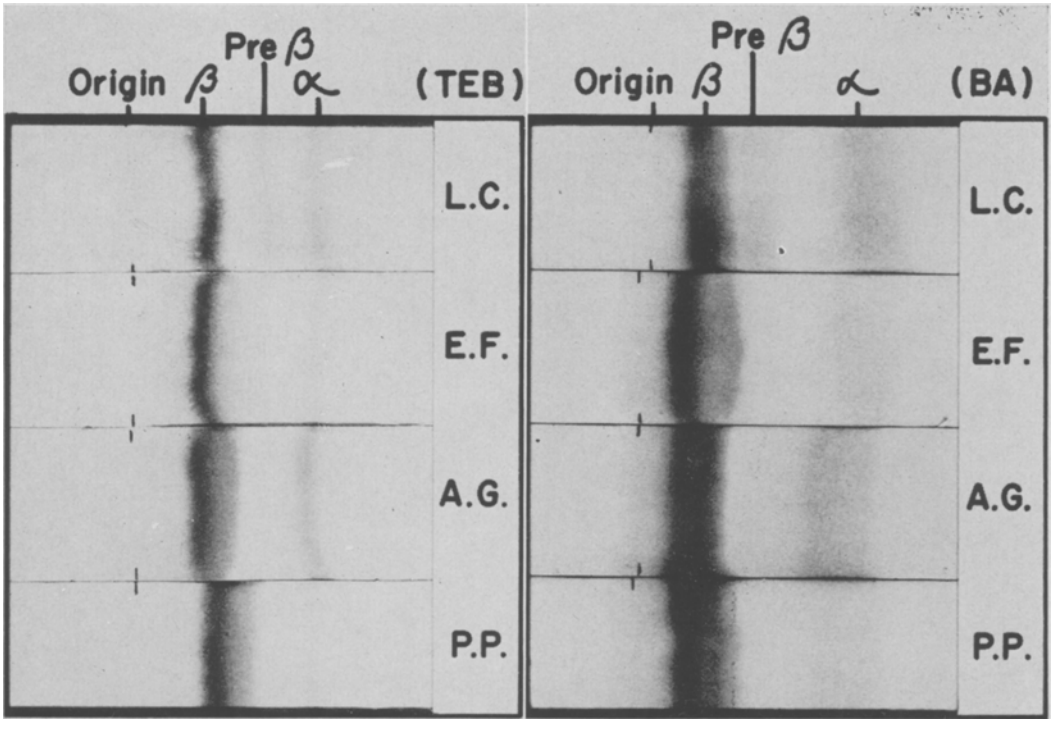

FIG. 1. Serum lipoprotein patterns developed in two different buffers simultaneously for 16 hr. A (left). TEB buffer. Patients L. C. and E. F. show complete separation of pre-β lipoprotein whereas A. G. and P. P. show only partial separation. B (right). BA buffer. The same kind of separation is observed, but the α-band is not as sharp.

the procedure were the same as in the method of Jencks and Durrum (1). The modifications were as follows. The cells were sealed with masking tape during electrophoresis, and the stained strips after rinsing with water were dried in the staining racks instead of by blotting.

TEB Buffer and Comparison With Barbital-Albumin (BA) Buffer

In all comparative tests, electrophoresis was carried out simultaneously in TEB buffer and BA buffer. The latter buffer was prepared according to Lees and Hatch (2) using Beckman B-2 buffer (pH 8.6 ionic strength 0.075) and human crystalline albumin. In our hands the 0.075 ionic strength buffer was equally satisfactory as the 0.1 ionic strength buffer used by Lees and Hatch.

RESULTS

There was effective separation of β-, pre-β-, and α-lipoprotein fractions of blood serum in this electrophoretic system. A few typical examples are shown in Figures 1-3. Chylomicrons, if present, remained at the origin and thus were separated from the other lipoproteins.

Identification of the pre-β band was based on detection of the lipoprotein fraction obtained on a simultaneous run of the serum sample in BA buffer. As can be seen in Figure 1, samples from patients L. C. and E. F. showed complete separation of pre-β lipoprotein in both TEB and BA buffers, whereas in samples from patients A. G. and P. P., a partial separation was observed. The results from these four patients are typical of those obtained from other patients having pre-β-lipoprotein. Lipoprotein patterns with no pre-β fraction were essentially the same when serum samples were analyzed simultaneously in both buffers.

In all samples tested, TEB buffer effectively separated all the bands that were separated by the BA buffer. In addition, as illustrated in Figures 1-3, the α-band was consistently more compact when electrophoresis was carried out in TEB rather than BA buffer. These results with α-lipoprotein are in agreement with

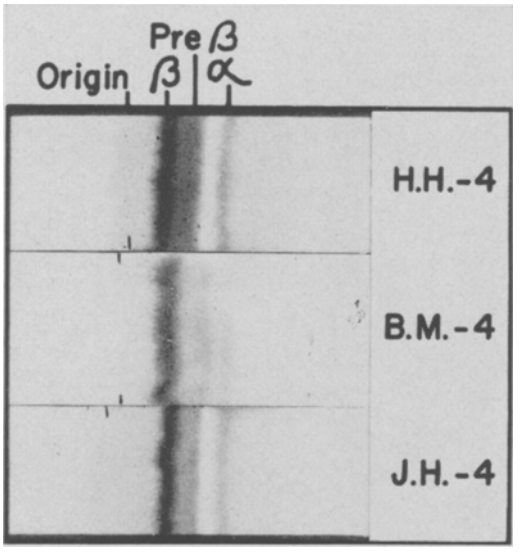

FIG. 2. Serum lipoprotein patterns developed in TEB buffer for 4 hr from three patients who show complete separation of pre-β lipoprotein.

the observations of Sonnino and Gazzaniga (3) with the TEB and barbital buffers.

Distinct separation of the lipoprotein components was attained in either 16 or 4 hr of electrophoresis (Fig. 1-3). Electrophoresis for 4 hr under constant voltage tended to sharpen all bands and made them more compact than the bands obtained after 16 hr of electrophoresis. Nevertheless, electrophoresis for 4 hr did decrease the mobility of the lipoprotein fractions (Fig. 3). However, the small R_f values of lipoproteins do not prevent the visual detection of the lipoprotein fractions. This is true even in cases where a chylomicron band is present in addition to the other fractions. The densitometric scans show complete separation of peaks. These small R_f values can be increased, in agreement with Morales-Malva et al. (5), simply by wetting the paper strips with 70% or 50% instead of the full strength buffer solution. However, in a number of samples the increase in mobility was accompanied by simultaneous diffusion of the bands. The lower the percentage of the buffer solution, the greater was the diffusion.

DISCUSSION

In the system described here, three factors contribute to the separation of lipoprotein fractions: TEB buffer, 52.8% alcohol solution as a solvent for Oil Red O, and the solvent-extracted paper strips.

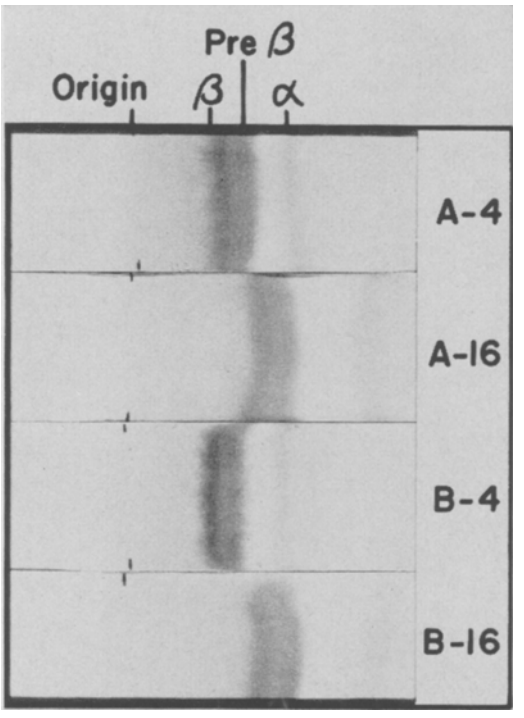

FIG. 3. Comparison of serum lipoprotein patterns from the subjects A and B, developed in TEB buffer for 4 and 16 hr.

There was a marked improvement in the staining when the Oil Red O was prepared in 52.8% alcohol solution. The lipoprotein bands were more intensely colored, and the background stain was much lighter in contrast to the strips stained with Oil Red O prepared in 60% ethanol. The drying of the strips in the staining racks is preferred because blotting often causes a blotchy appearance which interferes with scanning. Marked differences also were noted in the staining characteristics of paper strips. With some batches, the separated lipoprotein bands were diffused upon staining. But the same batches of paper strips, after extraction with solvents, showed more compact lipoprotein bands and a lighter and more evenly stained background. Commercially obtained paper strips are not of uniform quality, particularly with reference to the material in the paper that is stained with Oil Red O. Therefore, solvent extraction prior to electrophoresis makes this technique more reproducible.

The present electrophoretic system enables

the separation of pre-β from the β-fraction with concomitant separation of a compact and well-defined α-fraction without the use of albumin in the buffer.

Satisfactory resolutions can be obtained in 16 or 4 hr of electrophoresis. When the sample is subjected to 4 hr of electrophoresis, the procedure can be completed within 24 hr, but the 16 hr system requires 48 hr for completion. The two systems facilitate more efficient handling of the analyses, particularly if the samples are received in the laboratory at different hours of the day. Samples received in the morning may be subjected to 4 hr electrophoresis, and the rest may be included in the overnight or 16 hr run. The interpretation of the lipoprotein patterns obtained in both systems is the same.

ACKNOWLEDGMENTS

This work was a cooperative effort with the Heart Disease and Stroke Control Program, Regional Medical Programs, Health Services and Mental Health Administration, U.S. Department of Health, Education and Welfare. R. F. Witter, Chief of the Lipid Standardization Laboratory, revised the manuscript and E. B. Wylie of the National Communicable Disease Center provided the crystalline albumin.

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[Received August 26, 1968]

Inhibition of Cholesterol Synthesis by β-Benzal Butyric Acid and Derivatives[1]

G. PORCELLATI, D. GIORGINI and **E. TOJA**,[2] Department of Biochemistry, The University of Pavia, Pavia, Italy

ABSTRACT

Cholesterol biosynthesis has been examined using rat liver slices in vitro from 2-^{14}C-acetate and 2-^{14}C-mevalonate, in the presence of β-benzal butyric acid (BBA) and its metabolite, α-hydroxy β-benzal butyric acid (HBBA), both of which are postulated to act as potential hypocholesterolemic agents. Procedures have been devised to follow radioactivity incorporation of these precursors into the squalene, lanosterol and cholesterol fractions. The results show that cholesterol synthesis from labeled acetate is noticeably inhibited by BBA final concentrations as small as 10 μM, while the rate of labeling is much less inhibited by HBBA. When acetate is replaced by labeled mevalonate, cholesterol synthesis is hardly inhibited by both BBA and HBBA. The results indicate that BBA probably affects some of the reactions which lead acetate to mevalonate formation. Acetyl-CoA:ligase (E.C.6.2.1.1) and acetyl-CoA acetyl transferase (E.C.2.3.1.) therefore have been examined. Ligase activity is substantially inhibited only by 1 mM concentration of BBA and HBBA, whereas the transferase enzyme is unaffected. BBA probably affects other reactions in the metabolic sequence which converts acetate into mevalonate.

INTRODUCTION

In recent years, a considerable amount of research has been directed toward finding efficient inhibitors of sterol biosynthesis. As a result, many compounds have been found to inhibit cholesterol biosynthesis, both in vivo and in vitro.

The actions of various phenyl derivatives of 5-carbon branched chain acids have been evaluated by Canonica et al. (1). These products possess noticeable hypocholesterolemic effects, the most active compounds in this series being β-benzal butyric acid (BBA) (3-methyl-4-phenyl-3-butenoic acid, BBA) and 2-methyl-4-phenyl-butanoic acid (in vivo cholesterol biosynthesis inhibition and Triton hyperlipidemia test). But apart from some preliminary results (2), these derivatives have not been examined as in vitro inhibitors of sterol synthesis nor has their mechanism of action been elucidated.

In this paper an attempt has been made to obtain experimental evidence for the inhibitory effect of BBA and of α-hydroxy β-benzal butyric acid (HBBA) (2-hydroxy-3-methyl-4-phenyl 3-butenoic acid, HBBA) on sterol biosynthesis in vitro, and to report some indications on their site of action.

EXPERIMENTAL PROCEDURES

Biological Materials

Animals. Male Sprague-Dawley rats, weighing approximately 150 ± 25 g, were fed standard rat cubes and kept in identical hygienic conditions. The animals, fasted for 6 hrs, were killed in pairs by a blow on the back of the neck and the liver rapidly removed and placed into chilled Ringer solution.

Tissue Preparation. Liver slices, about 400 μ and weighing approximately 400 mg were prepared in the cold with a Stadie-Riggs microtome (A. H. Thomas, U.S.). Only the slices removed from the same separate liver block were distributed among the incubation flasks of the same experiment (about 20 slices per flask). They always possessed a constant weight to surface ratio value of 0.40–0.44 mg/mm^2 for all the experiments, and therefore a uniform value of the exchange surface was maintained.

Preparation of Enzymes and Assay of Enzymic Activity. A partially purified acetate-CoA ligase (E.C.6.2.1.1) was prepared according to Stern and Ochoa (3). The enzyme had a specific activity of 25 units/mg, as measured by the rate of sulfanilamide acetylation. One unit of activity equals 1 nmole of acetylated sulfanilamide formed per hour per milliliter. The enzyme could be stored for several days at −10 C without loss of activity.

[1] Presented in part at the International Symposium on Problems and Prospectives in Medical Therapy of Atherosclerosis, Parma, November, 1966.

[2] Present address: Polytechnic Institute, University of Milan, Italy.

Activity was normally assayed by incubating for 90 min under nitrogen at 37 C 20–40 mg enzyme protein in 2 ml volume of an incubation mixture consisting of: sufanilamide (0.5 mM), sodium bicarbonate (10 mM), potassium acetate (20 mM), Coenzyme A (2 mM), ATP (5 mM), magnesium chloride (5 mM) and phosphate buffer (40 mM, pH 7.40). After incubation, the enzymic transfer of acetyl group to sulfanilamide was determined according to the procedure of Chou and Lipmann (4).

Acetyl transferase (E.C.2.3.1.) reaction was followed with the same enzyme preparation by incubating 1 mM acetyl-CoA, in place of ATP, acetate and Coenzyme A, and by estimating the activity as explained previously. The enzyme was stable for two months at –20 C. Additional experiments have been carried out with a 65-fold purified acetyl-CoA acetyl-transferase (5) substantially free of acetate:CoA ligase activity. The enzyme was assayed in a final volume of 3.15 ml by incubating in a Beckman DU cuvette for 10 min at 37 C:0.125 μmoles acetyl-CoA, 0.3 μmole ρ-nitro-aniline, 15 μmoles potassium thioglycolate, 120 μmoles phosphate buffer (pH 6.80) and 0.80–0.85 mg of enzyme protein. The residual free *p*-nitroaniline was subsequently estimated by direct assay.

Chemicals and Labeled Substrates

Solvents. Benzene was washed with concentrated sulfuric acid, dried over calcium chloride and distilled. Chloroform, diethyl ether, pyridine, hexane, acetone, methyl alcohol and petroleum ether (bp 60–80 C) were freshly distilled over calcium chloride and stored under nitrogen. All the other solvents were not further purified.

Materials. BBA and HBBA were provided by Istituto Biochimico Italiano (Milan, Italy). BBA melted at 113 C, possessed a molar extinction coefficient of 15,600 at 246 mμ and four infrared maxima at 1700, 1250, 750 and 710 cm^{-1}. The elementary analysis was very close to the predicted values. Before using, both compounds were converted into the sodium salts, by addition of appropriate amounts of diluted sodium hydroxide.

Cholesten-5-en-3β-ol (cholesterol) and cholestan-3β-ol (dihydrocholesterol) were obtained from British Drug Houses; the first compound was crystallized via the dibromide (6), recrystallized with absolute ethanol and dried to constant weight under nitrogen. 5α-Cholest-5,7-dien-3β-ol (7-dehydrocholesterol) and 5α-cholest-7-en-3β-ol (Δ7-cholestenol) were obtained from Nutritional Biochemical Corp., Cleveland, Ohio. 4-Methyl-5α-cholest-7-en-3β-ol (methostenol), 4,4,14α-trimethyl-5α-cholesta-8,24 dien-3β-ol (lanosterol, contaminated with dihydrolanosterol) and squalene, were products of Mann Research Labs., Inc. Acetyl-CoA was from Sigma Chemical Co.; Silica Gel G, digitonin and 2,7-dichlorofluorescein from Merck, Darmstadt; silver nitrate from Carlo Erba, Milan.

Labeled Substrates. 2-^{14}C-acetate (27.4 mc/mmole), 2-^{14}C-mevalonate (3.77 mc/mmole) and 1-^{14}C-acetyl-CoA (54.5 mc/mmole) were obtained from commercial sources and diluted with inert substrates to a suitable specific activity. Before diluting, the N,N'-dibenzylethylenediamine salt of the mevalonic acid was converted to the potassium salt.

Incubation

Liver slices were incubated in Warburg flasks containing sodium chloride (118 mM), potassium chloride (4.7 mM), potassium dihydrogen phosphate (1 mM), magnesium sulphate (1.14 mM), sodium bicarbonate (3.56 mM), magnesium chloride (3.05 mM) and disodium hydrogen phosphate - hydrochloric acid buffer (9.2 mM, pH 7.40). The amounts of the precursors were as follows: 2-^{14}C-acetate, 9.20 μc, 0.58 mM (specific activity, S.A., 3.94), or 2-^{14}C-mevalonate, 4.80 μc, 0.32 mM (S.A., 3.77) or acetyl-CoA-1-^{14}C, 2 μc, 0.49 mM (S.A., 1.01). The Ringer solution was added before the slices and precursors, while BBA or HBBA were added last. The total volume was of 4 ml. All operations were carried out at 4 C. The flasks were flushed with O_2 and then stoppered and shaken in a water bath at 37 C for 2 hr at about 80 strokes/min.

Incubations with the enzymes were carried out as explained previously.

Isolation and Assay

General Procedures. At the end of the incubation period, the tissue samples were removed, washed by centrifugation with carrier substrate (60 μM) and twice with water, weighed and transferred into glass stoppered tubes. They were successively digested for 2 hr with N potassium hydroxide in 50% ethanol (5 ml) at 70 C, while flushed continuously with nitrogen. The unsaponifiable residue was extracted three times with 12 ml of petroleum ether (bp 60–80 C) and the combined clear extracts washed with distilled water, evaporated to dryness under nitrogen at 30 C and finally made up to appropriate volumes (generally 2 ml) in acetone-ethanol

(1:1 v/v) for purification by means of digitonin.

Prior to purification, a small aliquot was chromatographed in quadruplicate by thin-layer technique (TLC), according to Avigan et al. (7), on silica gel G pre-stained with 2,7-dichlorofluorescein. The UV-absorbing or fluorescent zones were: scraped from the plate for radioactivity assay, eluted for sterol estimation, radioautographed (Kodirex X film) and rechromatographed on silver nitrate-impregnated silica gel for further separation.

Chromatographic Methods. A preliminary separation of the sterols was carried out on standard plates using benzene-ethyl acetate (5:1 v/v) as the solvent (7). Plates were developed under nitrogen in the dark for 60 min. Normally, with 2-^{14}C-acetate, as the sterol precursor, only labeled cholesterol and small amounts of radioactive squalene and lanosterol could be identified. The same result was obtained, when chromatography was carried out on 40 cm plates (7) with benzene-ethyl acetate (20:1 v/v) as the solvent (24 hr. in the dark, under nitrogen). Small amounts of radioactive 7-dehydrocholesterol were sometimes present. When 2-^{14}C-mevalonate was the precursor, the last TLC technique revealed the presence of radioactive Δ7-cholestenol, large quantities of cholesterol (presumably contaminated with traces of cholestanol), 7-dehydrocholesterol, methostenol, lanosterol (probably contaminated with 24,25-dihydrolanosterol) and large amounts of squalene. Some overlap was present however between cholesterol, Δ7-cholestenol and 7-dehydrocholesterol.

Rechromatography was therefore carried out on the cholesterol spot separated on the 20 cm long plates, or on the three zones of cholesterol, Δ7-cholestenol and 7-dehydrocholesterol, separated on the 40 cm long thin layer plates and collected together. All the other spots were found to be homogeneous on further separation and were not rechromatographed. For rechromatography, the zones were accurately scraped from the plates, eluted into small sintered-glass columns twice with chloroform (6 ml) and then, with 3 ml of chloroform-methanol (98:2 v/v), evaporated and quantitatively rechromatographed on silver nitrate-impregnated silica gel plates (8) with chloroform-acetone (95:5 v/v) as the solvent. The plates were run in the dark, under nitrogen, for 90–100 min at 4 C and then lightly sprayed with 0.2% 2,7-dichlorofluorescein in absolute ethanol. A clear separation of the sterol components of the original cholesterol spot was obtained, and precise identification of labeled 7-dehydrocholesterol, cholesterol and Δ7-cholestenol carried out. Traces of labeled cholestanol were found by this procedure and by the technique of Truswell and Mitchell (9). Rechromatography was always carried out in triplicate, and the spots were removed for sterol determination, taken for radioactivity estimation, and radioautographed. The recovery of each of the sterols after rechromatography was over 85%.

Sterol Determination. The amount of sterol was measured on the purified digitonides and on chromatographic spots according to Zlatkis et al. (10). Standard sterols were used for quantitation. This was particularly important, for the color yield of 7-dehydrocholesterol was much lower than that of other sterols under similar conditions. Elution of the TLC spots was carried out according to Zeitman (11). The dichlorofluorescein dye was not extracted by this procedure. The recovery of standards from TLC plates approached 100%. When estimation was carried out on the silver nitrate plates, the Zak et al. (12) procedure was used by replacing the ferric chloride reagent by ferric nitrate to avoid cloudiness due to the silver chloride. Recovery was between 90–95%.

Radioactivity Measurements. Radioactivity was determined by liquid scintillation counting on the purified digitonides, or after elution from the Kieselgel with methanol (13). The dichlorofluorescein dye and the silver nitrate did not interfere with the counting in a way as to hinder or alter the incorporation data. Readings were properly corrected for any quenching effect. The ^{14}C counting efficiency was over 78% and the recovery of the label from the TLC plates approached 100%. The identity of the ^{14}C-lipids was confirmed by cochromatography with authentic lipid and cocrystallization, lanosterol and cholesterol as the acetate derivatives and squalene as the hexahydrochloride.

Calculation of the Results. Results have been normally calculated either as μmoles of cholesterol formed per hour per milligram of cholesterol (Fig. 1 and Tables I and II) or as μmoles of product formed per hour per gram of fresh liver tissue(Tables II and III). For calculation, in the first case, the μc of ^{14}C of the isolated cholesterol are divided by the S.A. of the precursor × milligrams of the tissue cholesterol × hour of incubation × C, whereas, in the second case, the μc of the

product are divided by the S.A. of substrates × gram of tissue × hour of incubation × C. C represents the number of molecules of precursor which are comprised in 1 molecule of cholesterol. For 2-^{14}C-acetate, C = 15; for 2-^{14}C-mevalonate, C = 5. In Figure 1, values are actually expressed as percent inhibition levels (control levels = 0).

RESULTS

In all the series of experiments, the rate of labeling was measured only for those components which were definitely identified, substantially homogeneous, and which were detected in measurable amounts. Therefore, after acetate incorporation, determinations were carried out only on the cholesterol spot isolated on the 40 cm long plates or on the purified labeled digitonide precipitate. The two procedures gave always comparable results. Radioactivity was occasionally assayed on the squalene spot. After the mevalonate uptake, countings were done on the squalene and lanosterol spots, both isolated on the 40 cm long plates, and on the cholesterol, Δ7-cholestenol and 7-dehydrocholesterol zones, isolated by the silver nitrate impregnation method. In these last experiments (controls and experimentals), labeling was slightly higher in the cholesterol digitonide precipitate than in the isolated cholesterol spot.

Biosynthesis of liver cholesterol was reduced by incubating the liver slices with labeled acetate in the presence of various concentrations of BBA (Fig. 1). Noticeable inhibition occurred at a 50 μM concentration, but at lower levels of inhibitor (10 μM) the acetate incorporation also differed from that of the controls. All experimental points were statistically significantly different from controls, except those at 1 μM concentration of BBA, owing to the larger scatter of the results. The experiments reported in Figure 1 were carried out by measuring the rate of labeling of the cholesterol spot isolated by TLC. No significant differences were observed, at any value of BBA concentration, between the results obtained by carrying out estimations on the purified labeled digitonide precipitate, or on the finally isolated cholesterol spot.

The conversion of radioactive acetate to cholesterol by rat liver slices in the presence of HBBA is reported in Table I. A comparison of the normal values shows that cholesterol is less rapidly synthesized from acetate in vitro at high concentration of the BBA

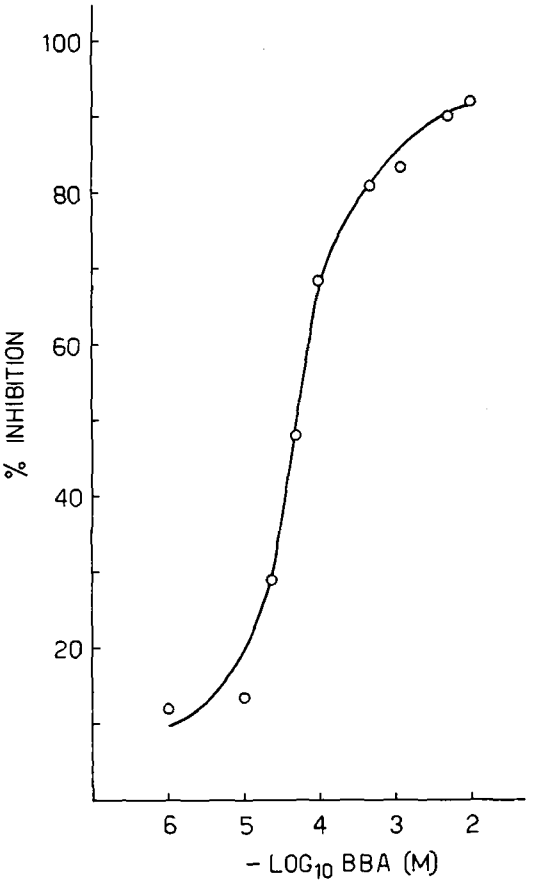

FIG. 1. The inhibition of liver cholesterol biosynthesis by β-benzal butyric acid in vitro. Incubation of liver slices and isolation of cholesterol were carried out as described in the text. Precursor: 2-^{14}C-acetate. The results, expressed as per cent inhibition values (control levels = 0), were calculated as μmoles of cholesterol formed per hour per milligram of cholesterol (mean control values ± S.E.M. of 0.83 ± 0.15, in eight experiments). For calculation, see the text. Six experiments were carried out for each value of BBA concentration.

derivative only. Thus, 5 mM HBBA inhibits cholesterol formation by 34% and the very high 10 mM concentration affects the metabolic conversion by over 55%.

In a second series of experiments, the inhibition of the 2-^{14}C-mevalonate incorporation into cholesterol by BBA and HBBA was examined (Table II), and the results were compared with those obtained with the acetate incorporation experiments. Mevalonate incorporation was decreased to about 80% of the control value only at 5 mM concentration

TABLE I

The Effect of α-Hydroxy-β-Benzal Butyric Acid (HBBA) on Liver Cholesterol Biosynthesis in vitro[a]

HBBA (mM)[a]	nc/mg Cholesterol	Activity[b]	% Inhibition	P
— (9)	100	0.85 ± 0.12		
0.05 (3)	108	0.91		
0.1 (6)	97	0.82 ± 0.13		
1.25 (3)	102	0.86		
5.00 (6)	66	0.56 ± 0.06	34	< 0.001
10.00 (5)	43	0.36 ± 0.04	58	< 0.001

[a] Incubation and TLC analytical procedures were carried out as described in the text. Precursor: 2-^{14}C-acetate. Numbers in parentheses indicate number of experiments in each group.

[b] Activity expressed as μmoles cholesterol formed per hour per milligram of cholesterol ± SEM, when specified. See text, for calculation. For some experiments, only the mean values are shown, owing to the small number of samples examined.

of BBA, lower levels being inactive in this connection. A further increase in the degree of the inhibition appeared at the very high concentration value of 10 mM. Although labeling was slightly higher throughout if determinations were carried out on the cholesterol digitonide precipitate, rather than on the isolated cholesterol spot, the relative degrees of inhibition by BBA were strictly comparable.

HBBA did not inhibit liver cholesterol synthesis from mevalonate in vitro at any concentration (data not shown in Table II).

Table II also shows that the degree of inhibition of cholesterol synthesis produced by BBA is similar to that of its precursors, squalene, lanosterol and Δ7-cholestenol. The result may thus signify, although not necessarily, that when inhibition occurs, no further effect is being observed along the metabolic steps occurring between squalene and cholesterol. The same result was observed, when acetate replaced mevalonate, as sterol precursor. In Table III the degrees of inhibition of squalene and cholesterol rates of synthesis, produced by BBA, are in fact reported, and appear to be similar.

According to our results it may be supposed that BBA, and to a much lesser degree HBBA, inhibits cholesterol synthesis from acetate, but hardly from mevalonate. Experiments have been performed, therefore, on the effect of BBA and HBBA on enzymes catalyzing the conversion of acetate into mevalonate; more precisely on the acetate:CoA ligase (E.C.6.2.1.1) and acetyl-CoA acetyl transferase (E.C.2.3.1.). The results (Table IV) show that BBA and HBBA inhibited the activation of the free acetate to acetyl-CoA at concentration values ranging from 1 to 20 mM. The enzyme was however not affected at concentrations lower than 1 mM which, on the contrary, were very effective as inhibitors of sterol synthesis from labeled acetate (Fig. 1 and Table III). In parallel experiments in vitro no inhibition of the acetyl-CoA acetyl transferase activity (see Experimental Procedures) was found with concentrations of BBA and HBBA up to 20 mM.

TABLE II

The Effect of β-Benzal Butyric Acid (BBA) on Sterol Synthesis From 2-^{14}C-Mevalonate in Rat Liver Slices[a]

	Rate of Labelling			
BBA (mM)[b]	Cholesterol[c]	Squalene[d]	Lanosterol[d]	Δ_7-Cholestenol[d]
— (8)	14.7 ± 1.10	6.42 ± 0.51	1.98 ± 0.26	1.56 ± 0.20
0.05 (3)	13.9	7.52	2.42	2.20
0.1 (6)	15.7 ± 1.12	7.06 ± 0.60	—	—
1.25 (3)	14.5	7.14	2.86	1.70
2.50 (4)	14.1	7.10	—	—
5.00 (6)	11.3 ± 0.91 (23%)	3.84 ± 0.40 (40%)	1.14 ± 0.18 (42%)	0.82 ± 0.12 (48%)
10.00 (3)	8.56 (42%)	3.90 (40%)	1.02 (48%)	0.80 (49%)

[a] Incubation was carried out as described in the text. The squalene and lanosterol rates of labeling were determined after isolation of the compounds by TLC on the 40 cm long plates (7); those of the cholesterol and Δ_7-cholestenol after rechromatography and isolation on silver nitrate impregnated plates. Owing to the faintness of its area, values for the rate of labeling of the isolated 7-dehydrocholesterol had a large scatter and results were unreliable.

[b] Number in parentheses indicate number of experiments in each group. For some experiments, only the mean values are shown, owing to the small number of samples.

[c] Figures indicate μmoles of cholesterol formed per hour per milligram of cholesterol (see text for calculation). Degree of inhibition in parentheses.

[d] Figures indicate μmoles per hour per gram of fresh liver (mean values). For calculation, see the text. Degree of inhibition in parentheses.

TABLE III

The Effect of β-Benzal Butyric Acid (BBA) on Squalene and Cholesterol Labeling From 2-^{14}C-Acetate in Rat Liver Slices[a]

BBA	(mM)[b]	Rate of Labelling Cholesterol[c]		P	Squalene[c]		P
—	(8)	1.61 ± 0.19			0.080		
0.01	(6)	1.40 ± 0.15	(13)	< 0.01	0.066	(15)	< 0.1
0.05	(6)	0.81 ± 0.10	(50)	< 0.1	0.040	(50)	> 0.1
0.50	(6)	0.30 ± 0.04	(81)	< 0.1	0.013	(83)	< 0.1
1.25	(6)	0.27 ± 0.02	(83)	< 0.001	—		
5.00	(6)	0.18 ± 0.015	(89)	< 0.001	—		
10.00	(6)	0.15 ± 0.015	(91)	< 0.001	traces		

[a] Incubation was carried out as described in the text. Activity of squalene was determined after isolation of the compound by TLC on the 20 cm long plates (7), and that of cholesterol after digitonine treatment and purification. For other experimental details, see the text.

[b] Number in parentheses indicate number of experiments in each group.

[c] Figures indicate μmoles per hour per gram of liver tissue (mean values ± SEM, when specified). For calculation, see text. Owing to the low content of radioactivity and to the small number of samples examined, for the squalene experiments only the mean values are shown. Degree of inhibition between parentheses.

DISCUSSION

A combination of chromatographic procedures and of precipitations has allowed us to carry out an adequate separation of most of the sterols which are found labeled after exposure of rat liver slices for 2 hr to radioactive acetate or mevalonate.

The results presented above clearly show that liver slices possess decreased capacities to convert acetate into cholesterol in the presence of small amounts of BBA. A 50% inhibition of the overall process is produced, in fact, by a 50 μM concentration of the drug and becomes almost complete at higher BBA levels. It is worth mentioning at this point that previous work (14) has shown little uptake of the ^{14}C-labeled-BBA by the rat liver slices in vitro under optimal conditions. The final concentrations of BBA, capable of lowering cholesterol biosynthesis in vitro, might therefore be even smaller than those indicated in the present work.

HBBA is a much less active inhibitory agent of sterol synthesis than BBA and is definitely inactive when synthesis is examined from labeled mevalonate. It is worth mentioning that HBBA has been shown to be a metabolite of BBA in animals and man (15,16). The hydroxylated compound might be considered, in this connection, a detoxication product arisen from BBA when this drug is metabolized in vivo. Taking into account these considerations, it could be useful to compare the present results with experiments carried out on the inhibition of sterol synthesis in animal species injected with BBA or HBBA.

The present results show that cholesterol synthesis from mevalonate is much less efficiently inhibited by BBA than from acetate. The results suggest that the drug acts along the enzymic steps which occur between acetate and mevalonate. Thus, the inhibition brought about by BBA and HBBA on the acetate:CoA ligase might be of some interest. The levels of BBA which inhibit the enzyme are, however, far greater than those capable of lowering sterol synthesis from acetate. This last consideration might indicate that other enzymic activities are affected by BBA along the metabolic sequence of reactions from acetate to mevalonate. This indication is indirectly confirmed by the fact that HBBA, which affects the acetate:CoA ligase to a similar extent, does not inhibit sterol synthesis at low concentrations as BBA does.

These considerations have gained further

TABLE IV

The Effect of β-Benzal Butyric and α-Hydroxy-β-Benzal Butyric Acids on the Acetate : CoA Ligase Activity of Pigeon Liver[a]

Inhibitor	(mM)	Number of experiments	Activity[b]	% Inhibition
—	—	4	27.1	—
BBA	0.1	3	27.8	0
BBA	1.0	3	23.8	12
BBA	5.0	3	15.2	44
BBA	10.0	2	11.4	58
BBA	20.0	2	8.7	68
HBBA	1.0	3	24.4	10
HBBA	5.0	3	17.1	37
HBBA	10.0	3	11.9	56

[a] For experimental details, see the text.

[b] Figures indicate μmoles acetylated sulfanilamide/mg enzyme protein/hr. Because of the small number of experiments, only the mean values are shown.

support by some preliminary studies carried out by us on acetyl-CoA incorporation into sterols in the presence of BBA. By overcoming the inhibition of the acetate:CoA ligase, we have shown that cholesterol synthesis from labeled acetyl-CoA is still noticeably inhibited by the phenyl derivative. It will be worth studying, therefore, the effect of the drug on other enzymic activities connected with mevalonate synthesis, particularly the β-hydroxy β-methyl glutaryl-CoA reductase. With the present experiments we have already shown that another enzymic activity, linked to mevalonate synthesis (acetyl-CoA acetyl transferase), is not inhibited by either BBA or HBBA.

The present results also show that the intermediate steps between squalene and cholesterol are probably unaffected by BBA and HBBA and, further, that inhibition of sterol synthesis from mevalonate is scanty. The sequence of the enzymic reactions occurring between mevalonate and squalene would appear to constitute a desirable site of inhibition of cholesterol synthesis in vivo, since it seems unlikely that intermediates in this step would accumulate as a consequence of the inhibition. BBA does not seem, however, to act upon these metabolic reactions. In this connection, it could be of interest to mention that 2-methyl 4-phenyl-butanoic acid, another hypocholesterolemic agent (1), seems to possess these properties (17).

ACKNOWLEDGMENTS

The work was supported in part from the Consiglio Nazionale delle Ricerche, Rome. B. V. Frattini and U. Valcavi (Milan) provided the β-benzal butyric and α-hydroxy β-benzal butyric acids, and M. C. Zapponi carried out determinations of acetyl-CoA ligase and acetyl-CoA acetyl transferase activity.

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[Received May 31, 1968]

Structure of Bovine Milk Fat Triglycerides: II. Long Chain Lengths[1]

W. C. BRECKENRIDGE and **A. KUKSIS**, Banting and Best Department of Medical Research and the Department of Biochemistry, University of Toronto, Toronto, Canada

ABSTRACT

The long chain triglycerides of bovine milk fat were isolated by thin layer chromatography, and their chemical structure determined by combined thin layer and gas liquid chromatography, and a stereospecific analysis of a molecular distillate of butteroil of comparable composition. The milk fat fraction (39% of total) contained C_8-C_{20} fatty acids which were distributed among the glycerides of 40-56 acyl carbon atoms in a manner not unlike that found for the same acids in the short chain triglycerides. Although individual triglycerides were not identified, the specific distribution of the fatty acids could best be accounted for by assuming a common pool of long chain 1,2-diglyceride precursors from which the bulk of both short and long chain triglycerides are synthesized by a stereospecific introduction of C_4-C_{18} fatty acids in position 3 of sn-glycerol. This hypothesis is compatible with the results of stereospecific analyses of the short and long chain fractions and of the total butteroil. It is supported by the nonrandom distributions demonstrated for the molecular weights of the milk fat triglycerides of different degrees of saturation.

INTRODUCTION

Both plasma lipids and de novo synthesis in the udder are known to contribute fatty acids to the synthesis of milk fat (1). Previous studies (2) would appear to exclude the possibility that the plasma triglycerides are incorporated intact into the milk fat. Precursor triglycerides must therefore be broken down at least partially to allow for a reesterification with fatty acids of both short and long chain lengths (3). Since the identification of the partial glyceride precursors might yield information about the mechanism of milk fat assembly, there has been much speculation regarding the potential intermediates (4-6). The experimental findings have been limited to a demonstration of specific association (7,8) and positional placement (9,10) of fatty acids in the short chain triglycerides, and a general non-randomness in the molecular weight distribution of the triglycerides in milk fat (11).

In the present study a detailed investigation has been made of the molecular association and positional and overall distribution of fatty acids in the long chain triglycerides. The data have been compared to the results of similar earlier analyses of the short chain triglyceride fraction and appropriate inferences have been made.

MATERIALS AND METHODS

The chemical reagents, solvents, chromatographic materials and analytical standards were as described (8,10). The long chain triglyceride fractions of milk fat were as obtained (8) during the isolation of the short and medium chain length triglycerides. Another long chain triglyceride fraction of milk fat was obtained as a residue by molecular distillation of butteroil (12). This material (D-3; 50% of total butteroil) was used for the stereospecific analyses.

Thin Layer Chromatography

The long chain triglycerides were isolated as outlined for the short and medium chain lengths (8). Plates of Adsorbosil-3 (20×20 cm, 250μ thick) were prepared by standard methods, and 5-10 mg of milk fat applied per plate as a band. The bands were developed in heptane-isopropyl ether-glacial acetic acid (60:40:4 v/v/v) and the lipid bands located by spraying with dichlorofluorescein. The triglycerides were recovered by elution with 5% methanol in diethyl ether. The long chain triglycerides were resolved on the basis of degree of unsaturation using similar plates made up of silica gel G containing 10% silver nitrate (8). About 5-10 mg of triglyceride was applied per plate and the plate developed with 0.65% methanol-chloroform (v/v). The bands were located and the lipids recovered as above.

Gas Liquid Chromatography

Triglycerides were analyzed on Aerograph 204-1B Dual Channel Gas Chromatograph (Varian-Aerograph, Walnut Creek, California). The instrument was equipped with dual col-

[1]Presented in part at the AOCS meeting, Philadelphia, October, 1966.

umns (stainless steel tubes, 2 ft x ⅛ in. OD, packed with 2% JXR on Gas Chrom. Q, 100-120 mesh). The columns were conditioned and operated as previously described (11) The chromatographic system was calibrated with mixtures of standard triglycerides, tributyrin through tristearin.

The fatty acids were determined by GLC of their butyl esters (1) using an F & M Model 402 Gas Chromatograph (F & M Scientific Corporation, Avondale, Pennsylvania) equipped with dual glass columns (4 ft x ¼ in. OD) containing 15% diethylene glycol succinate (DEGS) on 60-80 mesh Gas Chrom. P. Samples containing C_4-C_{18} fatty acids were determined by temperature programming from 70-220 C at 4 C/min. Long chain fatty acids were determined isothermally at 200 C. The instrument was calibrated with mixtures of standard butyl esters prepared by transbutylation of known amounts of high purity triglycerides (tributyrin through tristearin).

Values obtained from duplicate analyses of both standard triglycerides and fatty acid butyl esters showed a relative error of less than 3% for any peak comprising more than 10% of the sample, and less than 6% for any peak comprising less than 10% of the sample.

Stereospecific Analyses

The stereospecific examination of the long chain triglycerides of butteroil was performed essentially as described by Breckenridge (10), except that proportionally smaller amounts of material were used. The mixed 1,2- and 2,3-diglycerides were prepared from 1 g of fat. The lipolysis was stopped when approximately 50% of the total triglyceride had been hydrolyzed (4 min). The diglycerides were recovered by extraction with diethyl ether following dilution of the reaction mixture with methanol. They were purified by TLC on Adsorbosil-3 using the above described solvent system. About 40 mg of the phosphatidyl phenol was used for hydrolysis with phospholipase A.

RESULTS AND DISCUSSION

Preliminary Resolution

The resolution of milk fat triglycerides into short (SCT), medium (MCT) and long (LCT) chain triglycerides by TLC on plain silica gel was described in an earlier communication from our laboratory (8), as were the further analyses of the SCT and MCT fractions. The preparations of LCT obtained by this method contained triglycerides with 40-56 acyl carbons per molecule, and made up 38.7% and 39.8% of the total milk fat triglyceride. It should be noted that the LCT fraction contains several triglyceride groups with the same carbon numbers as the SCT and MCT fractions, but that these triglycerides do not possess identical fatty acid compositions. Thus in the SCT the C_{40} glycerides are largely comprised of 18,18,4, whereas in the LCT these must have been made up of combinations such as 16,14,10 or 16,18,8 since no butyric or caproic acids are present. The MCT contains both types of C_{40} triglycerides in appreciable amounts. This resolution of triglycerides within a molecular weight is due to differences in the polarity of these compounds and must have been operative also during the TLC of the C_{42}-C_{46} triglycerides which are represented in significant amounts in both MCT and LCT.

The preliminary segregation of butteroil triglycerides by molecular distillation is characterized by a separation based on differences in molecular weight only (12). Hence the triglycerides of corresponding carbon number in the various molecular distillates would be expected to contain identical fatty acid compositions. Therefore, while the overall triglyceride distributions were comparable, the distillation residue contained significant amounts of butyric and caproic acids, in contrast to the two preparations of LCT made by TLC. A removal of the more polar short chain triglycerides from the distillation residue prior to the stereospecific analysis was not necessary.

Combined TLC-GLC Analysis of LCT

Resolution of the long chain triglycerides on thin layers of silicic acid impregnated with silver nitrate gave a pattern more complex than anticipated for the relatively simple triglyceride mixture. Two bands were obtained for the monoenes and the dienes with two monoenoic and one saturated fatty acid per molecule. This was due to the presence of small amounts of elaidic acid in these primarily oleic acid-containing glycerides. Since oleic and elaidic acids are not readily resolved by the GLC of their butyl esters the *trans*-monoenes and the *trans*-dienes were pooled with the corresponding *cis*-isomers for the presentation of data. No differences were noted between oleic and elaidic acids in their association with other fatty acids in the glyceride molecules.

The GLC patterns of the triglycerides of the

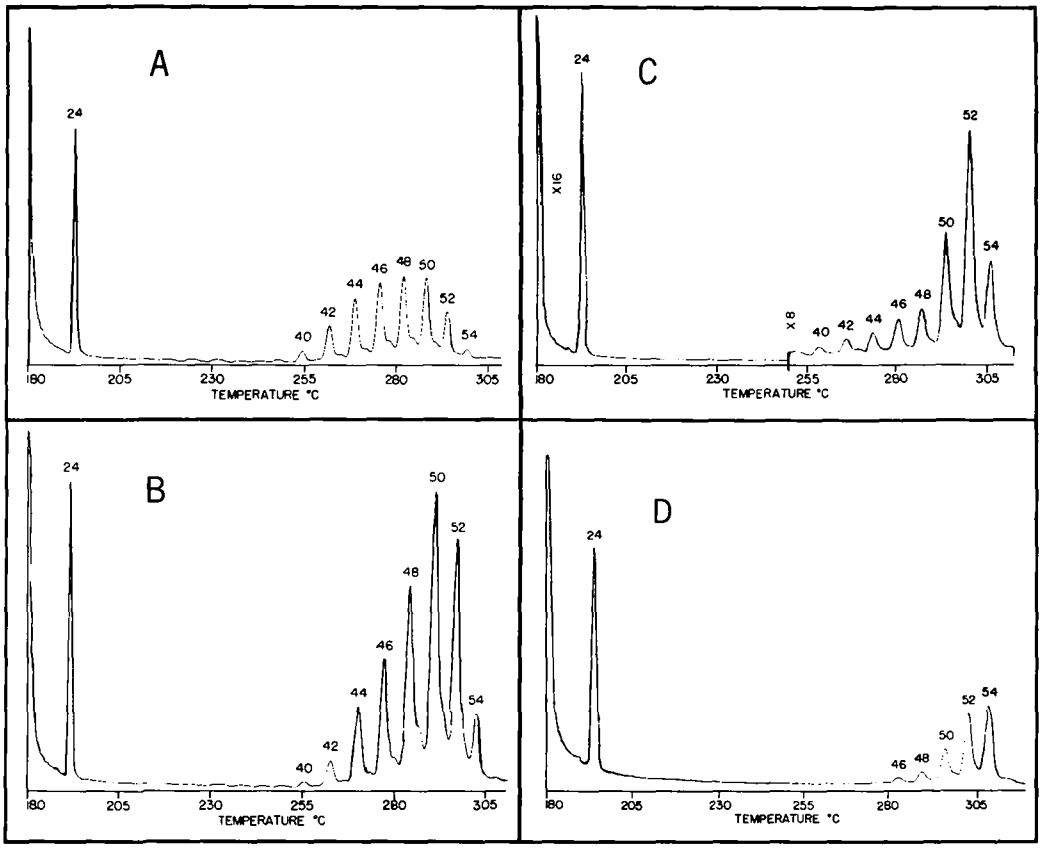

FIG. 1. GLC of long chain triglycerides of various degrees of unsaturation; A, saturates; B, monoenes containing one *cis*-monounsaturated acid; C, dienes containing two *cis*-monounsaturated acids; D, trienes containing three monounsaturated acids and dienes containing one linoleic acid residue. Temperature program as shown. Other operating conditions as stated in Ref. 8. Peak 24, trioctanoin used as internal standard.

saturates, monoenes, dienes and trienes are shown in Figure 1. As noted previously with the short and medium chain fractions (8), segregation of the saturated and unsaturated triglycerides by argentation TLC greatly improves the GLC resolution of adjacent even and odd carbon number peaks. It allowed a better estimate of the triglycerides of odd carbon number than was possible by GLC of the total milk fat triglycerides.

The composition of the triglycerides and the fatty acids of the various classes of saturation of the long chain fractions are shown in Tables I and II. In the saturates, which comprise 16.5% of the LCT, the major fatty acids are myristic (17.7%), palmitic (39.4%) and stearic (25.4%), while the major triglyceride types are C_{44} (13.9%), C_{46} (17.2%), C_{48} (16.4%) and C_{50} (15.6%). This suggests a broad range of triglycerides involving all the combinations of the saturated fatty acids in the sample. A small amount (1.4%) of tristearin is also present. In the monoenes (36.7% of the fraction) the major acids are palmitic (30.6%), stearic (17.3%) and oleic (30.6%), while the major triglycerides are C_{48} (17.6%), C_{50} (25.3%) and C_{52} (18.4%). The diene band (27.7%) made up of two monoenoic and one saturated fatty acid contained palmitic (16.0%) and oleic (59.3%) as major acids and C_{52} (37.1%) as major triglyceride. This suggests the occurrence of the 16,18:1, 18:1 triglyceride. The dienes made up of two saturated acids and linoleic acid migrated with the trienes comprised of three monoenoic acids and together accounted for 12.9% of the long chain fraction. The major acids were palmitic (18.5%), oleic 45.8%) and linoleic (13.2%), while the major glycerides were C_{52} (31.5%) and C_{54}

TABLE I
Composition of Long Chain Triglycerides of Bovine Milk
(moles %)

	Saturates[b]		Monoenes[b]		Dienes[b]		Trienes[b]		Polyenes[b]		Recovery[c]	Original[d]
TG[a]	Band	Total	Band	Total	Band	Total	Band	Total	Band	Total	Total	Total
38	0.3	—	—	—	—	—	—	—	—	—	—	—
40	3.0	0.5	0.4	0.2	0.7	0.2	—	—	—	—	0.9	1.0
41	0.4	0.1	—	—	—	—	—	—	—	—	0.1	—
42	8.3	1.4	2.3	0.8	1.5	0.4	—	—	—	—	2.6	3.0
43	1.8	0.3	0.6	0.2	—	—	—	—	—	—	0.5	0.5
44	13.9	2.3	6.8	2.5	2.6	0.7	0.7	0.1	—	—	5.6	5.9
45	3.5	0.6	1.1	0.4	—	—	—	—	—	—	1.0	0.7
46	17.2	2.8	11.1	4.1	5.6	1.5	3.5	0.5	2.4	0.2	9.1	9.3
47	4.0	0.7	2.0	0.7	0.4	0.1	—	—	—	—	1.5	1.2
48	16.4	2.7	17.6	6.4	7.8	2.2	8.1	1.0	5.6	0.4	12.7	13.5
49	3.4	0.6	3.5	1.3	1.7	0.5	—	—	—	—	2.4	2.0
50	15.6	2.6	25.3	9.3	19.9	5.5	17.7	2.3	15.2	0.9	20.6	21.5
51	2.6	0.4	4.0	1.5	4.4	1.2	—	—	—	—	3.1	2.2
52	8.0	1.3	18.4	6.8	37.1	10.3	31.5	4.0	26.7	1.6	24.0	24.5
53	0.2	—	1.8	0.7	2.2	0.7	—	—	—	—	1.4	1.0
54	1.4	0.2	5.0	1.8	15.6	4.3	36.2	4.7	41.6	2.6	13.6	13.0
56	—	—	—	—	0.6	0.1	2.1	0.3	7.1	0.4	0.8	0.7
58	—	—	—	—	—	—	—	—	—	0.1	0.1	—
	100.0	16.5	100.0	36.7	100.0	27.7	100.0	12.9	100.0	6.2	100.0	100.0

[a] Triglycerides identified by total number of acyl carbon atoms.

[b] Saturates, monoenes, dienes, trienes and polyenes are triglyceride types containing 0, 1, 2, 3, >3 double bonds per triglyceride molecule.

[c] Values obtained by proportional summation of the triglyceride types differing in degree of saturation.

[d] Values obtained for the sample before fractionation.

(36.2%). The polyenes (6.3%) contained oleic, linoleic and linolenic acids in various combinations with each other and with one saturated fatty acid to give largely C_{52} (26.7%) and C_{54} (41.6%) triglycerides. Tables I and II also compare the values reconstituted for

TABLE II
Fatty Acid Composition of Long Chain Triglycerides of Bovine Milk
(moles %)

	Saturates[b]		Monoenes[b]		Dienes[b]		Trienes[b]		Polyenes[b]		Recovery[c]	Original[d]
FA[a]	Band	Total	Band	Total	Band	Total	Band	Total	Band	Total	Total	Total
8:0	1.3	0.2	—	—	—	—	—	—	—	—	0.2	0.2
10:0	4.8	0.8	2.0	0.7	1.4	0.4	—	—	—	—	1.9	2.3
12:0	5.9	1.0	3.0	1.1	2.1	0.6	0.7	0.1	2.4	0.2	3.0	2.7
14:0	17.7	2.9	10.0	3.7	6.5	1.8	4.6	0.6	9.3	0.6	9.6	9.8
14:1	—	—	—	—	2.1	0.6	0.8	0.1	1.1	0.1	0.8	0.8
15:0[e]	3.1	0.5	1.3	0.5	0.5	0.2	—	—	—	—	1.2	1.0
16:0	39.4	6.5	30.6	11.2	16.0	4.4	18.5	2.4	19.7	1.2	25.7	24.4
16:1	—	—	2.8	1.0	5.2	1.4	5.4	0.7	3.6	0.2	3.3	3.2
17:0[e]	2.4	0.4	2.4	0.9	1.5	0.4	1.6	0.2	0.7	—	1.9	1.8
18:0	25.4	4.2	17.3	6.4	5.4	1.5	7.1	0.9	8.0	0.5	13.5	15.7
18:1	—	—	30.6	11.2	59.3	16.4	45.8	5.9	32.0	2.0	35.5	35.4
18:2	—	—	—	—	—	—	13.2	1.7	—	—	1.7	1.5
18:3	—	—	—	—	—	—	—	—	11.5	0.7	0.7	0.6
20:2	—	—	—	—	Trace	Trace	2.3	0.3	9.4	0.6	0.9	0.6
20:3	—	—	—	—	—	—	—	—	1.5	0.1	0.1	Trace
20:4	—	—	—	—	—	—	—	—	0.8	—	—	—
	100.0	16.5	100.0	36.7	100.0	27.7	100.0	12.9	100.0	6.2	100.00	100.00

[a] Fatty acids identified by the number of carbon atoms in the fatty acid residue and number of double bonds.

[b] Saturates, monoenes, dienes, trienes and polyenes are triglyceride types with 0, 1, 2, 3, >3 double bonds per glyceride molecule.

[c] Values obtained by proportional summation of the fatty acid composition of the triglyceride types differing in degree of saturation.

[d] Values obtained for the fatty acid composition of the sample before fractionation.

[e] Consists of normal and iso-branched acids.

TABLE III

Bovine Milk Fat Composition (moles %)

Triglycerides			Fatty Acids		
Carbon No.[a]	Recovery[b]	Original[c]	Carbon No.[d]	Recovery[b]	Original[c]
26	0.1	0.3	4:0	9.4	9.8
28	0.6	1.2	6:0	4.6	4.9
30	1.2	1.5	8:0	1.8	2.2
31	0.1	Trace	10:0	3.2	4.0
32	2.6	2.6	12:0	3.6	3.4
33	0.2	0.1	14:0	10.1	10.2
34	5.3	5.6	14:1	0.4	Trace
35	0.8	0.4	15:0[f]	1.4	1.4
36	10.4	11.3	16:0	23.7	23.4
37	1.3	0.6	16:1	2.7	1.7
38	14.4	15.5	UNK[g]	0.1	—
39	1.0	0.5	17:0[f]	1.3	1.5
40	12.1	11.8	18:0	10.0	12.5
41	0.6	N.D.[e]	18:1	24.5	22.4
42	6.6	6.7	18:2	2.2	1.6
43	0.4	N.D.[e]	18:3	0.6	1.0
44	4.9	4.8	20:2	0.4	Trace
45	0.5	N.D.[e]			
46	4.7	5.0			
47	0.6	N.D.[e]			
48	5.5	6.4			
49	0.9	N.D.[e]			
50	8.3	9.7			
51	1.2	N.D.[e]			
52	9.5	10.2			
53	0.5	N.D.[e]			
54	5.4	5.8			
56	0.3	Trace			

[a] Triglycerides identified by total number of acyl carbon atoms.

[b] Values obtained by proportional summation of the components after fractionation of the sample on thin layers of silicic acid impregnated with silver nitrate.

[c] Values obtained by an analysis of the sample before fractionation.

[d] Fatty acids identified by total number of carbon atoms and number of double bonds.

[e] Not determined.

[f] Consists of normal and iso-branched acids.

[g] Identity not determined.

the triglycerides and fatty acids from the subfractions with the results of the analyses of the original LCT fraction. The good agreement between these data indicates that the chromatographic fractionations were not accompanied by extensive losses of any specific molecular species.

Comparable compositions of fatty acids for the long chain triglycerides of butteroil have been reported by Blank and Privett (13). They found approximately the same amount of fully saturated glycerides with nearly identical fatty acid composition. While the fatty acid complements of the monoenes and dienes were also quite similar, the proportions of the two unsaturation classes were different. According to Blank and Privett (13) the monoenes were much higher (57% vs. 36.7%) the dienes slightly lower and the trienes and polyenes

TABLE IV

Triglyceride Types of Bovine Milk Fat (moles %)

Types	Experimental	Random
Saturates	32.9	39.0
Monoenes	37.6	38.7
Dienes		
SMM[a]	16.4	12.7
SSD[b]	5.3	2.7
Trienes	5.4	5.0
Polyenes	2.4	1.9

[a] Triglycerides containing one saturated and two monoenoic fatty acids.

[b] Triglycerides containing two saturated and one dienoic fatty acid.

present only in trace amounts. Furthermore, the ratios of myristic, palmitic and stearic acids are very similar throughout all the classes of unsaturation in both studies, but the total amount of the saturated acids progressively decreases with increasing amounts of the unsaturated acids. These relationships suggest that the fatty acid combinations within each triglyceride group may remain relatively constant but that the overall amount of each unsaturation class may vary with the milk fat preparation.

Table III shows the reconstitutions of the triglycerides and fatty acids from the SCT, MCT and LCT fractions of bovine milk fat. These values compare very well with the results of the overall analyses of the original milk fat, and should therefore represent a valid description of the detailed distribution of the fatty acids in milk fat. The relative error of these estimates is ± 5% or less. Table IV compares the experimental and the random estimates of the various triglyceride types grouped on the basis of uniform degree of unsaturation. Although the dienes and trienes were not always cleanly resolved, reliable estimates of their proportions could be obtained from the fatty acid composition of the overlapping portions of the thin layer bands. While the amount of the saturates is slightly lower than the random value, and that of the dienes slightly higher, the estimates for the monoenes, trienes and polyenes approximate the values predicted by random distribution. Clearly, analyses at this low level of fractionation do not indicate any high degree of preference for the formation of any specific triglyceride types.

Table V compares the experimental and the random distributions of the triglycerides by molecular weight in the various saturation classes. For purposes of simplified presentation the values for the dienes, trienes and polyenes were combined. It can now be seen

TABLE V
Triglyceride Distribution of Bovine Milk Fat (moles %)

	Saturates		Monoenes		Combined dienes, trienes, and polyenes	
TG[a]	Experimental	Random[b]	Experimental	Random[b]	Experimental	Random[b]
<26	....	3.0	....	....	....	....
26	0.4	3.5	....	1.6	....	....
28	1.7	3.3	0.1	1.7	....	....
30	3.1	3.0	0.6	1.3	....	....
31	0.2	0.2	....	....	....	....
32	5.4	4.3	1.8	1.7	0.3	0.5
33	0.5	0.4	....	....	....	....
34	11.8	6.0	3.2	2.1	0.7	0.9
35	1.9	0.7	0.3	....	....	....
36	19.8	11.3	8.9	5.2	1.7	0.9
37	2.2	1.2	1.5	0.5	....	....
38	16.6	10.9	19.4	10.9	5.4	3.2
39	1.2	0.9	1.5	0.8	0.3	....
40	9.3	6.9	13.4	9.8	13.6	10.9
41	0.8	0.7	1.0	0.3	....	....
42	5.7	7.0	6.5	6.2	7.5	5.9
43	0.6	0.7	0.6	0.3	....	....
44	4.2	4.1	5.2	5.7	5.1	3.6
45	0.8	1.0	0.6	0.5	0.3	....
46	3.8	5.3	5.2	8.0	4.8	5.0
47	0.8	1.5	0.9	1.0	....	0.5
48	3.2	8.2	7.1	10.9	5.6	6.4
49	0.7	1.5	1.4	1.8	0.7	0.5
50	3.0	5.2	9.7	14.5	12.6	14.5
51	0.5	1.0	1.5	1.8	2.2	1.8
52	1.5	2.8	7.0	10.3	21.8	25.4
53	....	0.2	0.7	0.5	0.7	1.4
54	0.3	0.5	1.9	2.6	15.3	18.6
56	....	....	....	....	1.7	....

[a] Triglycerides identified by number of acyl carbon atoms per molecule.

[b] Amount calculated from probability equations.

that the experimental distributions deviate greatly from those predicted by calculation. In all three classes of unsaturation the amounts of short chain triglycerides greatly exceed the values predicted by random association of the fatty acids, while the long chain glycerides are present in lower proportions. In this respect these non-random patterns are similar to the non-random distributions of triglycerides noted for total butteroil (11).

Further resolution of these triglycerides could be obtained by preparative GLC, which, preceded by argentation TLC, would yield relatively simple mixtures of glycerides of uniform molecular weight and uniform degree of unsaturation. Since this method was unlikely to give the amounts of material required for a stereospecific analysis, the idea was abandoned for the time being in favor of a stereospecific analysis of a molecular distillate of butteroil with a fatty acid and triglyceride composition comparable to that of the LCT fraction.

Stereospecific Analysis

A stereospecific analysis of the long chain triglyceride fraction of butteroil (LCTB) was desirable for several other reasons. Thus, a comparison of the overall fatty acid composition of the butteroils analyzed by McCarthy et al. (12), Blank and Privett (13) and Pitas et al. (9) shows that these oils are remarkably similar as are the short (SCTB) and long (LCTB) chain triglyceride fractions prepared from them by TLC (13) and molecular distillation (12). This suggests that the preparations of butteroil possibly vary less in their composition than those of the milk globule fats. Furthermore, stereospecific analyses have already been reported for whole butteroil (9) and for the SCTB fraction obtained by molecular distillation (10). In addition, Blank and Privett (13) had reported the fatty acid composition of position 2 of the LCTB fraction and had made comparisons to the original oil.

Table VI gives the fatty acid composition of the three positions of the glycerol molecule (numbered relative to sn-glycerol-3-phosphate) for the LCTB fraction along with the composition previously reported for the SCTB (10) fraction and the whole butteroil (9). While there were differences in the fatty acid compositions of the original butteroils analyzed by Pitas et al. (9) and by the authors (10), the relative distributions of the major long chain fatty acids between the 1 and 2 positions are in good agreement in all three samples. Thus both capric and lauric acids are present in the highest concentrations in position 2, and about twice as much myristic is found in position 2 as in position 1, in all fractions. The amounts of palmitic acid (32.8-41.1%) are comparable in the two positions but much less of it occurs in position 3 (4.9-10.0%). Stearic acid is preferentially incorporated in position 1 in all cases, while oleic is only slightly higher in position 1 than in the other two positions. In contrast to the specific placement of the short chain acids in position 3 of the SCTB found by us (10), Pitas et al. (9) reported some short chain acids also in position 1 and 2. The latter acids might have been concentrated in the C_{26}-C_{30} triglycerides not present in the short chain distillate examined, as trace amounts of dibutyryl triglycerides have been reported to occur in milk fat by Nutter and Privett (7). The fatty acid composition of position 2 of the triglycerides of the whole butteroil and the LCTB fraction is also very similar to that

TABLE VI

Positional Distribution of Fatty Acids in Butteroil Triglycerides (moles %)

	Original			Position relative to sn-glycerol-3-phosphate								
				1			2			3		
FA[a]	SCT_B[b]	LCT_B[c]	Total[d]	SCT_B[b]	LCT_B[c]	Total[d]	SCT_B[b]	LCT_B[c]	Total[d]	SCT_B[b]	LCT_B[c]	Total[d]
4:0	18.3	1.5	11.3	....	....	5.0	....	....	2.9	53.9	4.5	43.3
6:0	7.5	2.2	4.8	....	....	3.0	....	....	4.8	24.3	6.6	10.8
8:0	2.0	1.6	2.3	....	Tr	0.9	0.9	Tr	2.3	5.1	4.8	2.2
10:0	3.5	2.9	4.2	0.9	1.2	2.5	4.3	2.8	6.1	5.3	4.7	3.6
12:0	3.1	3.5	3.9	3.1	1.7	3.1	6.5	3.9	6.0	–0.3	4.9	3.5
14:0	11.0	11.4	11.5	10.8	6.3	10.5	22.8	15.9	20.4	–0.6	11.8	7.1
14:1	1.0	Tr	....	0.5	Tr	....	2.4	Tr	....	–0.1	....	....
15:0[e]	1.8	3.8	....	2.3	1.9	....	4.4	3.7	....	–1.3	5.8	....
16:0	27.8	28.2	27.1	41.1	37.9	35.9	37.4	38.7	32.8	4.9	8.0	10.1
16:1	1.6	3.0	2.0	1.9	2.8	2.9	3.1	3.8	2.1	–0.2	2.4	0.9
17:0[e]	1.3	0.9	....	3.3	1.6	....	1.3	0.8	....	–0.7	–0.3	....
18:0	6.7	13.1	10.4	14.8	18.9	14.7	3.5	8.5	6.4	1.8	11.9	4.0
18:1	13.0	26.1	21.1	19.8	25.9	20.6	11.8	19.6	13.7	7.4	33.4	14.9
18:2	1.0	1.2	1.4	1.0	1.0	1.2	1.2	1.6	2.5	0.8	1.0	–0.5
20:2	0.4	0.5	....	0.5	0.8	....	0.4	0.6	....	0.3	0.1	....

[a] Fatty acids identified by number of acyl carbons and double bonds per molecule.

[b] C_{34}-C_{42} triglycerides isolated from butteroil by molecular distillation and analyzed earlier (10).

[c] C_{36}-C_{54} triglycerides isolated from butteroil by molecular distillation and described previously (12).

[d] Total milk fat triglycerides from butteroil as analyzed by Pitas et al. (9).

[e] Contains normal and iso branched chain fatty acids.

reported for these fractions by Blank and Privett (13) on the basis of pancreatic lipase hydrolyses.

Table VII gives the fatty acid composition of the 1,2-diglycerides of the SCTB, LCTB and the whole butteroil. These values have been derived by summing the fatty acids in positions 1 and 2 and dividing by two. Although the SCTB and LCTB fractions together account for only 80% of total butteroil, the 1,2-diglycerides appear to be very similar in their fatty acids. The greatest differences are in the content of capric and oleic acids in the diglycerides derived from the whole oil and the molecular distillates. This, however, could be attributed to the somewhat greater proportion of these acids in the lower molecular weight triglycerides in the more volatile distillates (R-1 and R-2, see Ref. 12) not subjected to the stereospecific analysis. Some of these and other minor discrepancies could have been due also to the slight differences in the original butteroils. In general, however, the 1,2-diglyceride compositions of the triglycerides of different molecular weights are similar. Since the SCT and LCT fractions derived from the milk globule fat by TLC possess overall compositions of fatty acids and triglycerides which are comparable to those of the SCTB and LCTB, it is likely that they also show similar positional associations of these acids.

The above results suggest the possibility that the 1,2-diglycerides could have been derived from a common pool during the biosynthesis of the milk fat. Such a hypothesis requires further testing by determining the molecular weight distribution of the 1,2-diglycerides, which is not possible when the phosphatidyl phenols are used as intermediates. Also, it would now appear experimentally feasible to pursue these studies using milk fats synthesized from appropriately labeled precursor lipids.

TABLE VII

Fatty Acid Composition of 1,2 Diglycerides (moles %)

FA	SCTB[b]	LCTB[b]	Total[b]
4:0	....	....	3.9
6:0	....	....	3.9
8:0	0.4	Trace	1.6
10:0	2.6	2.0	4.3
12:0	4.8	2.8	4.6
14:0	16.8	11.1	15.5
14:1	1.4	Trace	N.D.[c]
15:0	3.3	2.8	N.D.[c]
16:0	39.1	38.3	34.4
16:1	2.5	3.3	2.5
17:0	2.3	1.2	N.D.[c]
18:0	9.1	13.7	10.4
18:1	15.8	22.8	17.1
18:2	1.1	1.3	1.8
20:2	0.8	0.7	N.D.[c]

[a] Legends as in Table VI.

[b] Values determined by summation of the fatty acid compositions of positions 1 and 2 followed by normalization. Total values calculated from data of Pitas et al. (9).

[c] Not determined.

ACKNOWLEDGMENT

This investigation was supported by the Special Dairy Industry Board, Chicago, Illinois, the Ontario Heart Foundation, Toronto, Ontario, and the Medical Research Council of Canada. The National Research Council of Canada provided a bursary to W. C. Breckenridge.

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[Received July 26, 1968]

Lipids of Retina: I. Analysis of Gangliosides in Beef Retina by Thin Layer Chromatography

SHIZUO HANDA[1] and **ROBERT MAIN BURTON**, Department of Pharmacology and Beaumont May Institute of Neurology, Washington University School of Medicine, Saint Louis, Missouri 63110

ABSTRACT

Lipids were extracted from beef retina by chloroform-methanol (2:1); the gangliosides were removed from the total lipid extract by partitioning into water and chromatographing on thin layer plates coated with silica gel. The analytical methods are described for estimating ganglioside components, i.e., N-acetyl neuraminic acid, hexoses, hexosamine, sphingosine and fatty acids, in the presence of silica gel. Major gangliosides present in beef retina have been tentatively identified as follows: a ganglioside containing two N-acetyl neuraminyl groups but no hexosamine; two gangliosides containing two N-acetyl neuraminyl groups and one hexosamine; and a ganglioside with three N-acetyl neuraminyl moieties and one hexosamine.

INTRODUCTION

Retina is an integral part of the central nervous system and has a characteristic structure in which a number of elements of the nervous system exist in discrete layers. Lowry et al. (1) measured the neutral and complex lipid distribution at each histological defined layer of the retina employing their microtechniques. They suggested that the ganglioside would be present in the outer segment. Collins et al. (2), Sjöstrand (3) and Hörnhamer et al. (4) have reported their studies which defined a number of the lipid classes present in pig retina. These studies indicated that the ganglioside content of retina is less than 1% of the total lipids. More recent papers by Fleischer and McConnell (5) and Eichburg and Hess (6) have also examined the neutral and polar lipid classes present in beef retina. This paper reports our analysis of several gangliosides present in beef retina.

PROCEDURES

Materials

Beef eyes were obtained from the slaughter house within 3 hr after the animals were killed. Retina was isolated from these eyes as a sheet and washed thoroughly with cold, normal saline to remove the humor. Beef brain gangliosides were prepared by the Folch method (7) for use as reference compounds. The compound neuNGly→gal→glc-cer from horse red blood cells (8) and neuNGly→neuNGly→gal→glc-cer from cat red blood cells (9) were a gift from T. Yamakawa of the University of Tokyo. The characteristic Tay-Sachs ganglioside, galNAc→(neuNAc) gal→glc→cer, was a gift of L. Svennerholm of the University of Gottenburg.

Methods

Thin layer chromatography (TLC) was carried out employing plates (20 × 20 cm) coated with adsorbosil-1 (Applied Science, State College, Pennsylvania) with a silica gel thickness of 250 μ. These plates were activated by heating at 130 C for 90 min. The lipid samples were dissolved in chloroform-methanol (1:1) and applied to the thin layer plates with micropipettes to give a 1 cm streak at the origin. After the sample application was completed, layers of thick filter paper, e.g., Whatman 3 MM, 20 × 10 cm, were layered on the silica gel 15 cm from the origin and fixed in place with the aid of a glass plate, 20 × 3 cm, and metal clamps. The plate was placed in the developing chamber which had previously been equilibrated with the solvent. The paper was pushed out of the chamber through a slit between the chamber and the glass cover. This exposed end of paper increases the effective length of the chromatographic procedure. This procedure has been employed earlier by Eichberger et al. (10). Under these conditions the best separation of gangliosides occurred within 3 hr employing solvent systems (A), chloroform-methanol-water-aqueous ammonium hydroxide (15 N) (60:35:6:2 v/v) and (B) *n*-propanol-water (7:3 v/v) (10,11).

Resorcinol reagent was employed for the detection of neuraminic acid-containing glycolipids (12). Iodine vapor and the anthrone reagent were employed as non-specific methods for determining the position of all compounds on the developed thin layer plate. Similar methods have been employed by others, e.g., Suzuki and Chen (13).

[1] Present address: Department of Biochemistry, Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo, Japan.

Quantitative analysis of ganglioside separated on the thin layer plate. The glycolipids of beef retina were applied to the thin layer plates with the Radin-Pelick applicator (Applied Science) for quantitative analysis. The chloroform-methanol solution of the glycolipids was applied in 15 cm streaks repeating as many times as necessary to streak enough lipids for analysis. Appropriate glycolipid standards were always applied to these plates as reference compounds. After the plates had been developed, compounds were detected by iodine vapor and marked on the silica gel using a sharp needle point. After the residual iodine had sublimed at room temperature, spots marked by the needle point were carefully removed by scraping with a razor blade. The reference glycolipids, which had been left on the thin layer plate, were detected by spraying with either the resorcinol reagent or the anthrone reagent.

In general three equal aliquots of the glycolipids were run side by side on the same TLC plate. The silica gel scraped from the three tracts of the plates were used for determination of neuraminic acid, hexosamines, fatty acids, sphingosine and hexoses. One aliquot was analyzed for neuraminic acid by the method of Svennerholm (14) and a modification of Miettnien and Takki-Luukkainen (15) by adding 0.5 ml of water and 0.5 ml of the resorcinol reagent to the silica gel scraped from the plate. The test tube was heated at 100 C for 15 min, cooled in an ice bath, the chromophore extracted with 1 ml of butyl acetate-butanol (85-15) and the optical density measured at 525 mμ. Blank values employing silica gel without N-acetyl neuraminic acid were always determined. Extinction of these blanks was approximately 0.02. This method shows good linearity up to 40 μg/ml of N-acetyl neuraminic acid.

The second aliquot was hydrolyzed with 0.6 ml of 2 N hydrochloric acid in screw cap tubes at 100 C for 12 hr. Hexosamine was assayed by the procedure of Gatt and Berman (16). After the aliquot was hydrolyzed, 0.25 ml of acetylacetone reagent was added and the tube heated at 100 C for 20 min. Ethanol (0.5 ml) and Ehrlich's reagent (0.25 ml) were added, and the contents of the tube thoroughly mixed with a Vortex mixer. The extinction was measured at 530 mμ. The presence of the silica gel had no apparent effect on either the hydrolysis of the ganglioside or on the chromophore formation.

The third aliquot was subjected to methanolysis by adding 2 ml of dry 5% methanolic hydrochloride (w/v) to the gel and the tube was sealed and placed in an oven at 90 C for 13 hr. The methyl esters of the fatty acids were extracted with three 2 ml portions of petroleum ether. The petroleum ether layer was used for fatty acid analysis by gas liquid chromatography (GLC) using a 6 ft column containing 10% apiezon L on 60-80 W.

The methanol layer remaining after the fatty acid extraction was centrifuged to remove the silica gel, the silica gel washed with methanol, and the supernatant layers combined. The methanol was removed by a stream of nitrogen and the residue dissolved in 1 ml of methanol and again centrifuged to remove residual traces of silica gel. An aliquot of the supernatant, 0.25 ml, was used for estimating the sphingosine base present. The methanol solvent was evaporated and the residue dissolved in 0.5 ml of 0.5 N sodium hydroxide and the sphingosine base extracted into diethyl ether employing three 0.3 ml portions of ether. The ether layers were combined and the solvent removed by evaporation. The residue was heated in an oven at 100 C to insure complete drying. The sphingosine base was measured by the procedure of Robins et al. (19). The samples were reacted with 60 μl of 0.3 N hydrochloric acid, 60 μl of borate buffer at pH 10.0 and 12 μl of fluorodinitrobenzene. This mixture was heated to 60 C for 30 min. Next, 60 μl of 6 N hydrochloric acid and 210 μl of propionic acid were added and the contents of the tubes stirred with a Vortex mixer. The extinction was measured at 420 mμ.

The residual aliquot, 0.75 ml, from the methanolysis step was used for estimating hexose by the anthrone-sulfuric acid reagent (17). The methanol was removed from the sample by evaporating with a stream of nitrogen. The dried residue was dissolved in 0.1 ml of 85% phosphoric acid. One milliliter of anthrone-sulfuric acid reagent was added and the tube heated at 100 C for 15 min. The measurement was made at 625 mμ. Both glucose and galactose standards were used. After the ratio of glucose to galactose was determined by GLC, the molar concentrations of glucose and galactose was determined by solving simultaneous equations. Blank values were low and no effect of the silica gel on the hexose determination was apparent. In some experiments the hexoses released by methanolysis were converted to their trimethylsilyl derivatives and analyzed by GLC using the method of Yamakawa and Ueta (18), i.e., 2.5% SE-30 on gas-chrom Z. This procedure was employed to determine the ratio of galactose to glucose.

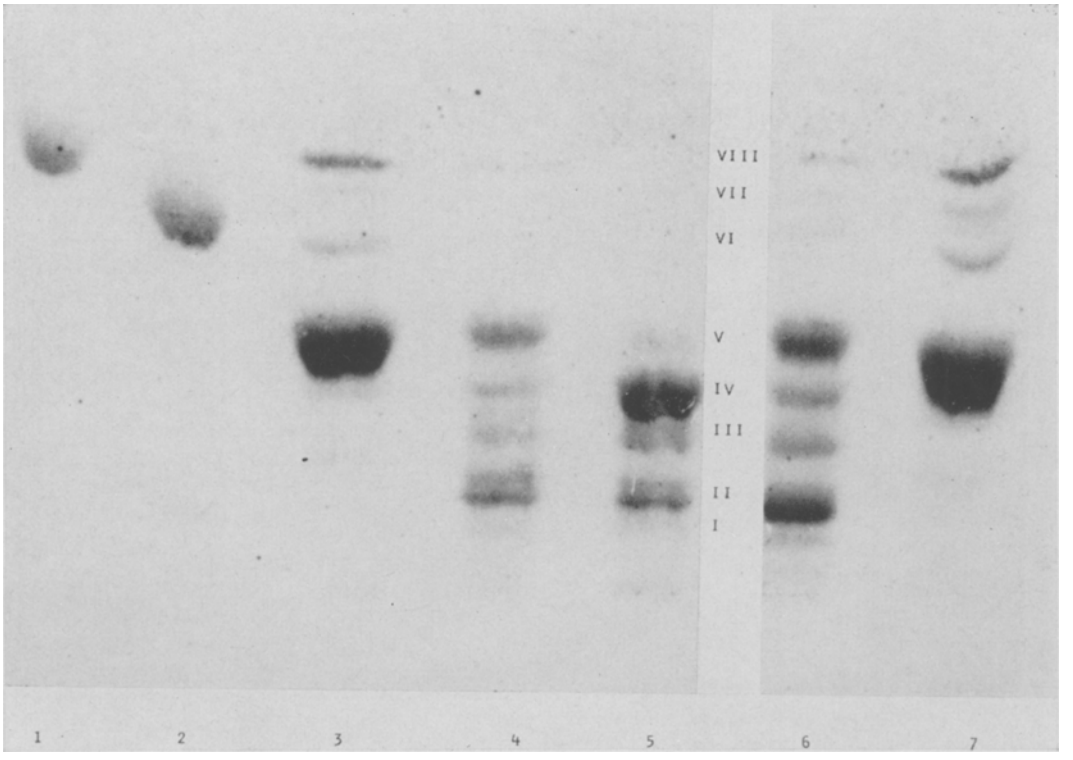

FIG. 1. Thin layer chromatogram of gangliosides. 1 = hematoside, i.e., neuNGly→gal→glc→cer., 2 = ganglioside from Tay-Sachs patient brain, i.e., galNAc→(neuNAc) gal→glc→cer; 3 = beef retina gangliosides eluted from silicic acid column with chloroform-methanol (6:4 v/v), major component is ganglioside V (C_1); 4 = beef retina gangliosides, total profile; 5 = beef brain gangliosides; 6 = beef retina gangliosides, duplicates of track 4 but twice concentration. 7 = beef retina gangliosides, duplicate of track 3. Solvent: chloroform-methanol-water-aqueous ammonium hydroxide (60:35:6:2 v/v). Spray: Bial's reagent, i.e., 0.1 g orcinol, 1.0 ml 1% ferric chloride, 40.8 ml 11.6 N hydrochloric acid, diluted to 50.0 ml with water. After spraying the TLC plate, it is covered with a glass plate and heated at 95 C for minutes.

RESULTS AND DISCUSSION

Beef retinas from 36 eyes were extracted and partitioned by the Folch method (7). The total lipid content was 28.2 mg/g of wet weight retinas or approximately 15.5 mg per retina. The aqueous layer obtained from the partitioning procedure was dialyzed against distilled water and lyophilized. The neuraminic acid content of the aqueous phase was 171 μg/g of wet weight retina, or 94 μg per retina. Thus, gangliosides are present in retina at 0.6% of the total lipid.

A typical TLC of beef retina ganglioside is shown in Figure 1, tracks 4 and 6. It may be seen that there are four distinct major glycolipids and four minor spots. The pattern of the glycolipid distribution differs from that of beef brain ganglioside, track 5, as reported by Wherrett and Cumings (12). For convenience in the laboratory these gangliosides have been designated by roman numerals I through VIII based on their mobilities as shown by the TLC in Figure 1. The four major gangliosides are those designated II, III, IV and V and have a neuraminic acid distribution respectively as follows: 26.4%, 22.6%, 18.7% and 32.3%. The components of these four gangliosides are presented by the data in Table I. The minor ganglioside which just precedes II was not identified. The analysis of the hexose content by GLC indicates a ratio of galactose to glucose of 2 for gangliosides, II, III and IV and a ratio of 1 for ganglioside V. The fatty acid composition was similar for all four gangliosides comprising 1.6% myristic acid, 37.0% palmitic acid, and 61.4% stearic acid. There was little detectable long chain fatty acids.

Silicic acid column chromatography of these gangliosides permitted the separation of ganglioside V from the other major gangliosides by

TABLE I
Beef Retina Ganglioside Composition

Ganglioside designation[a]	Molar ratios[b]					
	Ganglioside	Sphingosine	Neuraminic acid	Hexosamine	Hexose	Galactose glucose
II (C_4)	1.0	1.0	2.6	1.0	3.0	2
III (C_2)	1.3	1.0	2.2	1.0	3.4	2
IV (B_4)	1.1	1.0	2.3	0.5	3.3	2
V (C_1)	1.9	1.0	1.9	0.06	2.3	1

[a] Roman numerals refer to Figure 1; letter in parenthesis refers to ganglioside designation presented in "Lipid and Lipidosis" (21).

[b] Molar ratio referred to ganglioside C_4 as standard or to sphingosine as reference standard.

elution with chloroform-methanol (6:4). Ganglioside V was contaminated with the minor gangliosides VI, VII and VIII, (see Fig. 1, Track 3). Chloroform-methanol (1:1) eluted gangliosides I, II, III and IV.

From the data presented and the chromatographic behavior it may be concluded that II is a trineuraminyl-ganglioside, C_4, III is a dineuraminyl-ganglioside, C_2; IV is a dineuraminyl-ganglioside, B_4, but contaminated with V, and V is a dineuraminyl-ganglioside which does not contain hexosamine.

Ganglioside V was shown to chromatograph in an identical manner to neuNGly→neuNGly →gal→glc-cer obtained from cat red blood cells. The molar ratio of components in V is consistent with the identification of V as ganglioside C_1. Ganglioside VII chromatographed in the identical manner to that of Tay-Sach's ganglioside, i.e., galNAc→(neuNAc)gal →glc→cer. Ganglioside VIII chromatographed as hematoside, i e., neuNGly→gal→glu→cer. It is interesting to note that the cat red blood cell glycolipid and hematoside contain long chain fatty acids, i.e., primarily lignoceric acid, and N-glycolyl neuraminic acid, whereas the retinal glycolipids of similar composition contain stearic and palmitic acids and N-acetyl neuraminic acid. Ganglioside VI was not identified.

While the presence of neuNAc→neuNAc→ gal→glc→cer in brain tissue has been reported by Kuhn and Wiegandt (20), its presence in retinal tissue is of interest because it is present in twice the molar concentration of the other major gangliosides, accounting for one third of the glycolipid neuraminic acid. This explains the major difference between the ganglioside profiles of retina and brain tissues.

ACKNOWLEDGMENT

During the course of this work S. Handa was a Fulbright Scholar from Japan. This project was supported in part by a U.S. Public Health Service grant No. NB01575-10.

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[Received October 7, 1968]

Isoenzymes of Lipoxidase

SARA A. HALE, T. RICHARDSON, J. H. VON ELBE and **D. J. HAGEDORN**[1],
Department of Food Science and Industries, University of Wisconsin, Madison 53706

ABSTRACT

A polyacrylamide disc gel electrophoretic technique is described for studying isoenzymes or multiple molecular forms of lipoxidase in extracts of fresh green peas, pea seeds, wheat, fresh green beans and green bean seeds. After electrophoresis, gels containing starch were incubated with linoleic acid. Hydroperoxide bands were visualized by treating the gel with acidic potassium iodide. Staining specificity was established using the following criteria. Activity was absent when extracts or gels were boiled and when linoelaidic and oleic acids were used as substrates. Cyanide did not inhibit staining, whereas α-tocopherol, hydroquinone and nordihydroguaiaretic acid did. Prevention of the appearance of artifactual bands was attempted by: pre-electrophoresis of the gels; electrophoresis in the presence of thioglycollic acid; elimination of the large pore gel; pH adjustment of the plant extracts; and extraction of extract solids with lipid solvents. Gel patterns indicated two to three lipoxidase bands for fresh green peas and pea seeds, one to two bands for fresh green beans and green bean seeds, and four bands for wheat.

INTRODUCTION

Lipoxidase (E.C. 1.13.1.13) catalyzes the hydroperoxidation by molecular oxygen of *cis, cis*-1,4-pentadiene systems in unsaturated fatty acids such as linolenic, linoleic and arachidonic acids (1). Some evidence for the occurrence of two types of lipoxidases has been reported in peas and other legumes (2), one attacking only the free acid and the other attacking triglycerides in addition to the free acid. These different enzymes might possibly be detected as isoenzymes.

The term "isoenzyme" was proposed by Markert and Moller (3) to describe different proteins with similar enzymic activity. "Isoenzyme" is now recommended by the Standing Committee on Enzymes of the International Union of Biochemistry to describe the multiple enzyme forms occurring in a single species (4).

Although most of the work with isoenzymes has been for clinical purposes, few plant enzyme systems have been studied. Guss et al. (5) studied the lipoxidase isoenzymes in wheat and soybean. They observed two to four lipoxidase isoenzymes in wheat and four isoenzymes in soybean. These workers attributed differences in the oxidation of various substrates by unfractionated soybean and wheat lipoxidase to the existence of isoenzymes (6).

The present paper describes a polyacrylamide disc gel electrophoretic technique for studying lipoxidase isoenzymes in fresh green peas, pea seeds, wheat, fresh green beans and green bean seeds. Attempts are made to obviate artifacts by manipulating the electrophoretic conditions and by variously treating the extract.

MATERIALS AND METHODS

Extraction of Lipoxidase

Fresh peas. Four extracts of fresh peas were made: the first and second from peas purchased from a local commercial source, and the third and fourth from peas supplied by the Department of Plant Pathology, University of Wisconsin. Extracts three and four were of peas (*Pisum sativum* var. Early Perfection) from plants that were 50 and 55 days old, respectively. The extracts will be designated hereafter as I, II, III and IV.

For extracts II, III and IV, the fresh peas were ground thoroughly under nitrogen in a mortar and pestle with sand and a minimal amount of cold 12.5% (w/v) sucrose solution. The resulting slurry was stirred with a magnetic stirrer at moderate speed to minimize foaming for 30 min at 4 C under nitrogen. The slurry was strained through cheesecloth, and the resulting liquid was centrifuged at 4 C in a Beckman Model L-2 ultracentrifuge at approximately 62,000 × *g* for 30 min. The supernatant, which contained the enzyme, was frozen in approximately 10 ml aliquots. Lipoxidase activity in the frozen extracts remained evident in the polyacrylamide gel patterns for periods up to nine months. The peas used for extract I were placed in a beaker and frozen rapidly in a dry ice and acetone bath and were stored at −20 C before extraction.

Pea seeds. Whole pea seeds (*Pisum sativum*

[1]Department of Plant Pathology, University of Wisconsin, Madison.

var. Surprise) and whole bean seeds (*Phaseolus vulgaris* var. Gardengreen) were ground to a coarse powder in a Wiley mill. During the grinding, dry ice was added to the seeds as a coolant. Each powder was suspended in five volumes of cold acetone and centrifuged at 4 C for 15 min in an International clinical centrifuge. This solvent extraction was repeated twice, and then the powders were dried under vacuum at room temperature. The partially defatted powders were then extracted with 12.5% (w/v) sucrose solution as described above.

Polyacrylamide Disc Gel Electrophoresis

Electrophoresis on polyacrylamide gels was performed essentially by the method of Ornstein and Davis (7) except that the small pore solution number two contained 1% soluble starch for iodometry (Mallinckrodt analytical reagent) and was made fresh daily, as was the ammonium persulfate solution. The modified method of Davis (8), which did not include ferricyanide, was also used, but no differences in enzyme patterns were noted between the two methods.

Large volume of sample was necessary (0.1 to 0.2 ml of the extract) because of the low lipoxidase concentration in the extracts. Samples were layered on top of the large pore gel, or on top of the small pore gel in the experiments in which the large pore gel was omitted. Some experiments were also conducted in which the gels were subjected to electrophoresis prior to sample addition with 0.2 ml of thioglycollic acid (TGA) in 50% (w/v) sucrose solution (1 μmole thioglycollic acid/0.05 ml 50%, w/v, sucrose solution, pH adjusted to 8.5). Other samples were also electrophoresized with TGA-50% (w/v) sucrose solutions of various TGA concentrations layered on the gel before sample application. Under these conditions, the TGA migrated faster than the sample proteins. Pre-electrophoresis of the gels and the inclusion of TGA in the procedure were attempts to obviate any artifacts due to oxidation of proteins by residual ammonium persulfate (9).

Electrophoresis was performed at 4 C with a current of 3-5 ma per tube.

Staining Procedure

After electrophoresis, the gels were removed from the tubes and placed in 10×100 mm test tubes, and substrate solution was added to completely cover the gels. The substrate solution was prepared by ultrasonically dispersing linoleic acid (greater than 99% purity, Hormel) in 20 ml distilled water. Ultrasonification was carried out in an ice bath under nitrogen using a Branson sonifier model LS-75 for 3 min at a power level setting of eight and tuned to 9.5 amp. The dispersed linoleic acid was then diluted with 0.05 M Tris-HCl buffer at pH 8.3 to yield a final concentration of 2×10^{-3} M linoleate. The gels were in the substrate solution for 30 min at room temperature with frequent inversion to assure adequate aeration. In enzyme inhibition tests, appropriate concentrations of inhibitor were added to the substrate prior to incubation.

The gels were then removed from the substrate solution, rinsed thoroughly with distilled water, and placed in clean 10 × 100 mm test tubes. The tubes were then filled with the staining solution which consisted of 5 ml saturated potassium iodide solution per 100 ml 15% acetic acid. Dark brown to blue activity bands appeared within 2 to 20 min and were optimally developed within 30 min.

To prevent background staining due to autooxidation of the potassium iodide, the acetic acid solution was degassed by the application of vacuum prior to and after the addition of the potassium iodide. The saturated potassium iodide solution was prepared daily.

Thin-Layer Chromatography

A portion of the peas used for extract III was blanched for 4 min in boiling water and extracted according to the method of Allen et al. (10). The final chloroform layer was evaporated to dryness and redissolved in chloroform to yield a concentration of 300 μg of lipid/5 μliters.

Thin layer plates were coated with Silica gel G, and activated for 30 min at 180 C. Five microliters of sample or standard was applied to each spot. Solvent systems used were: petroleum ether (bp 30–60 C)-diethyl ether-glacial acetic acid (90:10:1) for neutral lipids and chloroform-methanol-water (65:25:1) for phospholipids. Standards used were fish oil triglycerides, dipalmitin (Hormel) and trimyristin (Eastman). Phospholipid standards were phosphatidyl ethanolamine, sphingomyelin and phosphatidyl choline prepared in this laboratory or obtained commercially from Applied Science Laboratory. Developing sprays were Rhodamine 6-G, Rhodamine B and 2,7-dichlorofluorescein, which are general lipid stains; Dragendorff's reagent, specific for phosphatidyl choline; and the spray of Dittmer and Lester (11), specific for phospholipids.

Extraction of Fatty Acids

An aliquot of extract II was dialyzed against distilled water at 4 C overnight to remove suc-

rose, and then pervaporated at 4 C to two thirds the original volume. Protein was precipitated with five volumes of cold acetone. The precipitate was resuspended two times in five volumes of cold chloroform-methanol (2:1) and once in five volumes of cold acetone. Centrifugation for 15 min at 4 C in an International clinical centrifuge followed each resuspension. The resulting flaky white precipitate was dried under a vacuum at room temperature and suspended in 1 ml of 12.5% (w/v) sucrose solution (pH adjusted to 8.5). Aliquots of the suspension were used for electrophoresis. The sample was stored in a screw-cap vial under nitrogen at 4 C until electrophoresis.

RESULTS AND DISCUSSION

Effect of Heat Treatment

Boiling of the crude extract until it gelatinized was necessary to eliminate all traces of activity on the gel. The enzyme could be inactivated after electrophoresis and before substrate incubation by placing the gel in boiling water for 15 min. Thus, the band patterns observed appear to be due to enzymic activity.

Staining Specificity

A series of experiments were undertaken with green pea and pea seed extracts to rule out the possibility of activity bands on the gels due to heme-catalyzed peroxidation.

No bands were evident with gels containing pea seed or fresh pea lipoxidase incubated in chromatographically pure oleic acid or with 99% pure linoelaidic acid (Sigma). Lipoxidase bands appeared only after incubation of the gels in linoleic acid. Such results are to be expected since lipoxidase is specific for the *cis, cis*,1,4-pentadiene system found in linoleic acid, but not in oleic or linoelaidic acid. In addition, if heme catalysis were evident, one might expect activity bands with oleic and, particularly, with linoelaidic and linoleic acids.

Heme-catalyzed peroxidation is inhibited by the presence of cyanide in the substrate solution. Cyanide, at a concentration of 10^{-3} M, had no effect on the band pattern for both fresh pea and pea seed lipoxidase.

According to Maier and Tappel (12), heme-catalyzed oxidation of linoleic acid requires an induction period of about 24 hr at less than 7.1 $\times$ 10^{-3} M linoleate concentrations. The linoleate concentration used routinely in this procedure is 2 $\times$ 10^{-3} M or lower than that required for rapid initiation of heme-catalyzed lipid peroxidation.

Tappel (13) has reported that lipoxidase is inhibited by α-tocopherol, hydroquinone and nordihydroguaiaretic acid (NDGA), with NDGA being the most potent inhibitor. Addition of hydroquinone at 10^{-3} M to the substrate for extracts II through IV reduced band intensity, although the gel darkened rapidly when placed in acidic potassium iodide. α-Tocopherol at 10^{-2} M was only partially soluble in the substrate solution, and thus decreased band intensity about 50% over a control for extracts II through IV. NDGA at 3 $\times$ 10^{-4} M completely inhibited lipoxidase activity; at 10^{-6} M, band intensity was inhibited approximately 50% over a control for extracts II through IV.

The above results indicate that the bands produced on the polyacrylamide gels under the conditions described are due to lipoxidase activity.

The same experiments—cyanide inhibition, substrate concentration, substrate specificity and inhibitor reactions—were carried out with green bean seed and fresh green bean extracts with substantially the same results. Thus, there appears to be lipoxidase isoenzyme activity in both fresh green beans and green bean seeds.

Effect of Method of Extraction

Extract I, for which the peas were frozen before extraction, gave no bands with the procedure described earlier. On the other hand, unfrozen peas yielded extracts which gave multiple band patterns upon electrophoresis. Possibly, freezing the peas altered the extractability of the lipoxidase since freezing *per se* did not alter the enzymic activity, as evidenced by lipoxidase bands in extracts kept frozen for nine months.

Foaming was avoided at all times in making the extractions. When extracts were allowed to foam excessively, smears were produced on the gels rather than the expected band patterns. Apparently, lipoxidase is easily denatured by foaming.

Preliminary Electrophoretic Studies of Isoenzymes

The number of isoenzyme bands indicated was 2 to 3 for fresh green peas and pea seeds, 1 to 2 for fresh green beans and green bean seeds, and 4 for wheat (var. Selkirk) break shorts. Most experiments were performed on fresh peas and pea seed extracts.

Figure 1 shows the band pattern for extract II of the fresh peas and the pea seed extract (gels 1 and 4). The presence of activity between the large and small pore gels is indicated by the arrow. Extracts III and IV gave a similar band pattern—origin activity, an inter-

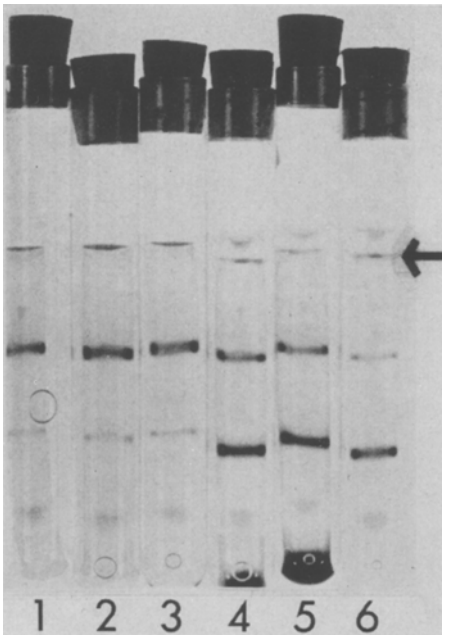

FIG. 1. Effect of TGA on isoenzyme pattern on polyacrylamide gels of fresh pea extract II and pea seed extract: 1, pea seed extract; 2 and 3, pea seed extract with TGA layered on gel prior to sample addition; 4, fresh pea extract II; 5 and 6, fresh pea extract II with TGA layered on gel before sample. Activity between large and small pore gels 1 through 6 is indicated by the arrow.

mediate band and a lower major band—except that the intermediate band did not always appear for either extract.

Effect of Thioglycollic Acid (TGA) and pH Adjustment of Sample

Several workers have noticed variability in band patterns when working with various isoenzyme systems. It has been suggested that free radicals in the gels could be causing these variations in band patterns (9,14,16). However, in an attempt to demonstrate free radicals in the gels, we examined the gels by electron spin resonance spectrometry. No free radicals could be observed either in the large or small pore gels.

Brewer (9) used 0.7 μmoles of TGA per gel dissolved in 50% (w/v) sucrose adjusted to pH 8.5, layered on the gel before the sample was applied to protect the sample proteins. Under these circumstances, the TGA migrates more rapidly than the protein. The use of a reducing agent such as TGA was an attempt to eliminate free radicals from the gels, as well as to protect the proteins from oxidation by residual persulfate in the gels. If free radicals or ammonium persulfate were present in the gels, they might cause polymerization of protein, resulting in multiple bands which might be misconstrued as isoenzymes.

Using Brewer's method, we either layered TGA on the gels before sample application or pre-electrophoresized with added TGA. When gels were used with a large pore gel and without pH adjustment of the sample, TGA had no effect upon the band patterns observed for pea seed extract or for fresh pea extract II, as shown in Figure 1. TGA did seem to have an effect, at times, upon mobility and band intensity when extracts III and IV of fresh peas were given the same treatment as above. In some experiments with extract III, the intermediate band was eliminated under the above conditions. In general, the band pattern for extract III was consistently a lower dark band in addition to origin activity, whereas an intermediate band appeared in approximately one experiment out of five. Extract IV always gave a lower dark band and origin activity similar to extract III and in approximately two out of three experiments an intermediate band. Activity always occurred between the large pore and small pore gels with extracts II, III and IV.

Possibly, the above anomalies might be due to a pH effect. The pH of the extracts was 5 to 6 and the pH of the running gel was routinely 8.5. In this case, a portion of the extract might become basic upon entering the running (small pore) gel, but there would be insufficient base to react with all the proteins. Hence, a portion of the enzyme would be more highly protonated at the outset and would tend to trail the more negative major band.

Also, interconversion of one isoenzyme to another might be occurring. Jacobson (17) has demonstrated the interconversion of faster-moving isoenzymes of *Drosophila* alcohol dehydrogenase to the slower-moving isoenzymes when absorbed on diethylaminoethyl cellulose; the reverse interconversion can be brought about in the presence of 0.05 M nicotinamide adenine dinucleotide. Kitto et al. (18) have observed malate dehydrogenase isoenzymes from chicken mitochondria which are interconvertible by iodine or acid treatment. Sodium borohydride treatment of an esterase from maize causes the appearance of enzyme species with altered electrophoretic migration rates (19). Jolley and Mason (20) have described the interconversion of multiple forms of mushroom tyrosinase by variation of pH, ionic strength and protein concentration. Such evidence tends to point to-

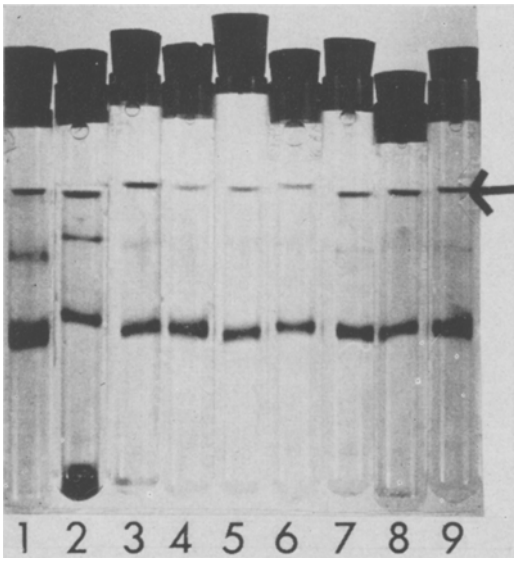

FIG. 2. Effect of pH adjustment of sample and deletion of large pore gel upon isoenzyme pattern on polyacrylamide gels of fresh pea extracts II, III and IV: 1, 2 and 3, fresh pea extract II; 4, 5 and 6, fresh pea extract III; 7, 8 and 9, fresh pea extract IV. The apparent upper band in gels 4 through 9 is a heavy concentration of unstained protein in the gel and not a lipoxidase activity band. Origin activity at tops of all gels is indicated by the arrow.

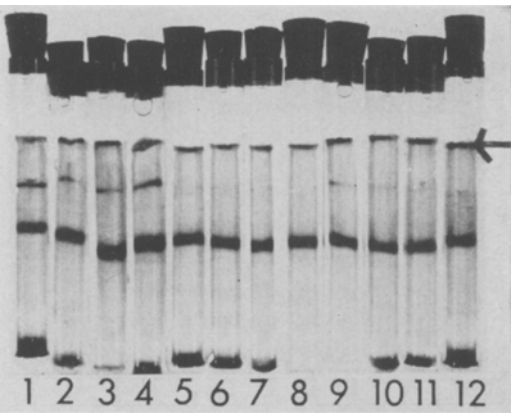

FIG. 3. Effect of pH adjustment of sample and deletion of large pore gel on isoenzyme patterns on polyacrylamide gels of fresh pea extracts II, III and IV: 1, 2, 3 and 4, fresh pea extract II; 5, 6, 7 and 8, fresh pea extract III; 9, 10, 11 and 12, fresh pea extract IV. The faint upper band in gels 9 through 12 is a lipoxidase activity band. Origin activity at tops of all gels is indicated by the arrow.

ward the existence of conformers—isoenzymes which change their conformation by the binding or release of modifiers (18). Modifiers could well be present in the form of TGA or some compound from the crude plant extract itself.

To test the effect of pH upon isoenzyme pattern with pea lipoxidase isoenzymes and also wheat break shorts lipoxidase isoenzymes, we adjusted the pH of the plant extracts to 8.5 and deleted the large pore gel (which routinely had a pH of 6 to 7, or lower than that of the running gel). Figures 2 and 3 show the results with pea lipoxidase isoenzymes. The intermediate band is absent in some cases.

Extract II was possibly from a different variety of pea than extracts III and IV. Many workers have found that isoenzyme patterns of the same enzyme differ between varieties (21). Hence, the difference in band patterns between extract II compared to III and IV is not unusual.

Fottrell (22) found, when separating legume root nodule esterase isoenzymes by starch gel electrophoresis, that a difference in age of four days between samples produced a different band pattern. Lanzani et al. (23), when working with peroxidase isoenzymes in wheat germ, found that the occurrence of different peroxidase isoenzymes in plants can be related to functional states of the tissues or to growing stages, or to pathological states or to the effects of radiation. Here again, our observed differences in band pattern are not unusual.

The intermediate band for extract IV, which appears in Figure 3 and not in Figure 2, may be due to a variation in concentration of enzyme applied to the gel.

Figures 4 and 5 show the results with wheat break shorts extract. Without pH adjustment of the extract before application to the gel and without deletion of the large pore gel (Fig. 4). the two major bands are on the bottom in relation to the two faint bands. Layering TGA on the gel before sample addition has no effect on the wheat break shorts extract pattern, as can be be seen in Figure 4. Thioglycollic acid was layered on gels 3 through 6 prior to sample addition.

Figure 5 shows the effect of pH adjustment and large pore deletion on band pattern observed with wheat break shorts extract. The two faint bands (indicated by the arrow) appear below the two major bands rather than above the two major bands as in Figure 4. Whether this difference is due to interconversion of the bottom set of bands to the top set of bands or modification of the relative mobilities of the

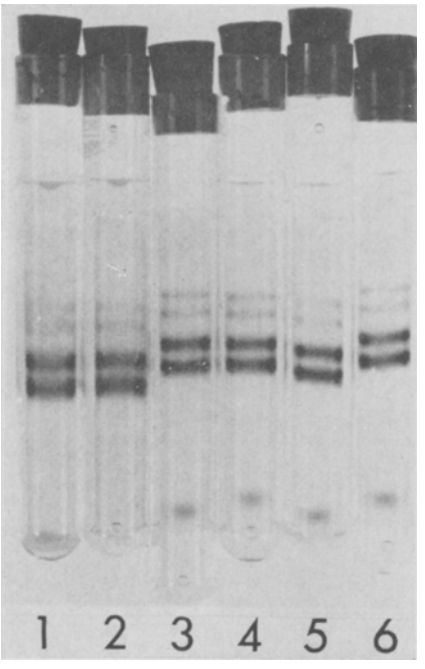

FIG. 4. Effect of TGA on isoenzyme patterns on polyacrylamide gels of wheat break shorts extract: 1 and 2, wheat break shorts extract; 3, 4, 5 and 6, wheat break shorts extract with TGA layered on gel prior to sample addition.

two sets of bands is not known.

In general, pH adjustment and large pore gel deletion gave more consistent band patterns with all extracts.

Effect of Extraction of Crude Pea Extract by Lipid Solvents

Lewis et al. (24) found that prolactin growth hormone, hemoglobin and myoglobin showed altered electrophoretic behavior when fatty acids were present. Muscle aldolase, which normally migrates as one band, gave at least four new bands when electrophoresized in the presence of oleic acid. Alteration of electrophoretic pattern occurred only under alkaline conditions.

We therefore attempted to extract free fatty acids from an aliquot of extract II as described previously. There was still a multiple band pattern similar to that of fresh pea extract II in Figure 1 (gel 4), suggesting that the bands we had been observing were not due to interaction of lipoxidase protein with free fatty acids. The band pattern was faint, probably because of the severe treatment given the extract. One week after extraction, no lipoxidase activity could be observed in the fatty acid-free extract. Hence, lipoxidase must have been partially denatured by the extraction procedure.

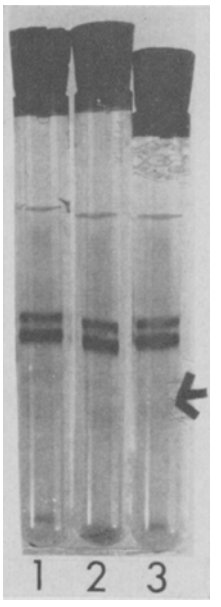

FIG. 5. Effect of pH adjustment and deletion of large pore gel on isoenzyme pattern on polyacrylamide gels of wheat break shorts extract.

Thin Layer Chromatography

Thin layer chromatography revealed the presence in fresh peas of diglycerides, triglycerides, phosphatidyl ethanolamine and a large amount of phosphatidyl choline. Future work should explore these lipids as possible substrates for isoenzymes of lipoxidase.

CONCLUSIONS

These data indicate that lipoxidase in fresh peas, pea seeds, fresh green beans, green bean seeds and wheat break shorts extracts exists in multimolecular forms or isoenzymes. The activity observed at the top of the small pore gels may be another isoenzyme or polymerized lipoxidase protein. Koch et al. (25) have reported the existence of a triglyceride lipoxidase in green peas and several species of beans. Perhaps one of the bands observed in this study is triglyceride lipoxidase. Further study is needed in this area.

Adjustment of the pH of the sample before application to the gel and deletion of the large pore gel in general gave more reproducible patterns with all extracts tested.

Adatthody and Racusen (26) have noted that both the pH and the ionic strength of the extraction medium played an important role

in the recovery of certain peroxidase isoenzymes. Future work should include a study of the effect of the extraction medium upon the lipoxidase isoenzymes extracted.

ACKNOWLEDGMENTS

Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This study was supported in part by a grant from Cooperative Research Service, United States Department of Agriculture.

Electron spin resonance measurements were made by Dr. S. Nelsen, Dept. of Chemistry, University of Wisconsin.

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[Received August 22, 1968]

Effect of Dietary Cholesterol on Bile-Acid Composition of Gall Bladder Bile From Guinea Pigs

JWU-SHENG TUNG and ROSEMARIE OSTWALD[1,2] Department of Nutritional Sciences
University of California Berkeley, California 94720

ABSTRACT

The composition of gall bladder bile acids from control and cholesterol-fed, anemic guinea pigs was analyzed by thin-layer-chromatographic and colorimetric techniques. In both control and cholesterol-fed animals, the gall bladder bile acids constituted about one third of the total bile solids. The main component of the bile acids of both groups of animals was chenodeoxycholic acid, which was predominantly conjugated with glycine. No cholic acid was present although this is the main bile acid in most mammals. The major difference in bile composition between control and cholesterol-fed animals was the conjugation pattern of chenodeoxycholic acid. The ratio of glycochenodeoxycholic to taurochenodeoxycholic acid was high, 6.4, for control animals, and decreased to 2.4 for the cholesterol-fed, anemic animals. Impaired liver function, limited availability of glycine, and greater efficiency of taurocholanic acids for the disposal of excess cholesterol may be involved in the mechanism for this phenomenon.

INTRODUCTION

The main catabolic pathway of cholesterol is the formation of bile acids in the liver. Glycine- or taurine-conjugated bile acids are excreted with the bile into the intestinal tract. Therefore, the changes in bile-acid composition, bile acid-conjugation pattern, or the rate of bile-acid excretion are highly significant to the metabolism of cholesterol.

Not all species react in the same manner to excess dietary cholesterol. Rabbits and chickens become severely hypercholesterolemic and develop atherosclerosis. In dogs and rats the influence of ingested cholesterol is much less marked (1), but an increased elimination of cholesterol as fecal bile acids has been found (2,3). The response of man to a cholesterol-supplemented diet is still a controversial subject. Wilson and Lindsey, (4) for instance, reported a moderate increase in the serum cholesterol level while Keys (5) found only slight effects. No consistent effects on either the biliary bile acid composition or the turnover of cholic acid have been demonstrated (6). Guinea pigs and rabbits develop a hemolytic anemia (7-11). In the guinea pig the anemia is accompanied by an increase in the amounts of unesterified cholesterol in plasma, red blood cells and adrenal glands. Other tissues including liver, spleen, heart, kidney and lungs show increases of both esterified and unesterified cholesterol as well as striking pathological changes (12-14).

The present investigation deals with the effects of a diet containing cholesterol on the composition and the pattern of conjugations of gall bladder bile acid of guinea pigs.

MATERIALS AND METHODS

Animals

The animals were mixed strains of young male guinea pigs (Dependable Animals Supplier, Martinez, California) weighing between 200 to 225 g at the start of the experiment. Composition of the basal diet is shown in Table I. To this diet was added 1% cholesterol for the experimental diet. The ingredients were mixed with water and pelleted. The dried pellets were stored at 4 C. The cholesterol-fed animals were considered anemic, and were autopsied when their red blood cell count had fallen below 4 x $10^6/mm^3$.

Bile Sample Preparation

The gall bladder bile was collected during autopsy, performed 20-24 hr after removal of food cups. Either bile or the gall bladder with its content was immediately frozen in dry ice, and lyophilized until a constant weight was obtained. The lyophilized bile samples were kept in a desiccator and stored in the freezer at -10 C until analyzed. It was extracted with chloroform-methanol (2:1 v/v) (20 ml solvent for 1 g of dry weight of sample) by boiling on a steam bath for 1 min (15). The solution was cooled to room temperature, centrifuged, and the residue

[1]Material in this paper has been submitted in partial satisfaction of the requirements for the degree of Master of Science in Nutrition in the Graduate Division of the University of California, Berkeley.

[2]A portion of this material was presented at the 7th International Congress of Biochemistry in Tokyo, Japan, August, 1967.

TABLE I
Composition of Basal Diet (12)

Component	g/100 g diet
Casein	30.0
Cornstarch	20.0
Cerelose (purified carbohydrate)	7.9
Cottonseed oil	10.0
Sucrose	6.5
Solka Flok (cellulose)	15.0
HMW salt mix (41)	6.0
Choline bitartrate	0.36
Potassium acetate	2.5
Magnesium oxide	0.5
Zinc carbonate	0.013
Water-soluble vitamin mixture	5.0
Fat-soluble vitamin mixture	5.0
Ascorbic acid	0.2
Water-soluble vitamin mixture	
Cerelose	2000
Thiamine HCl	6.4
Riboflavin	6.4
Pyridoxine	6.4
Ca-pantothenate	16
Niacin	80
Folic acid	4.0
Biotin	0.2
Vitamin B_{12} (1% in mannitol)	20
Fat-soluble vitamin mixture:	
Vitamin A palmitate (1×10^6 units per g)	6.96
Alpha-tocopherol	8.0
Menadione	0.8
Vitamin D_3	0.016
Cottonseed oil	2000

(coagulated protein) was washed with a small amount of solvent. The combined supernatants were washed with 0.2 volumes of water. Thin-layer chromatography (TLC) showed that this procedure separated the conjugated bile acids found in the aqueous methanol layer from the neutral lipids which were in the chloroform layer. Free bile acids were distributed in both layers.

Gallstone Analysis

Fresh gall bladder bile from anemic animals was separated from gallstones by centrifugation. The stones were then washed with distilled water, and lyophilized. Lipids were extracted with chloroform-methanol (2:1) under reflux, on a steam bath, for 1 hr. The extracts were filtered, and the residue was re-extracted with ethanol. The extracts were analyzed by the same methods used for bile extracts (see below).

Thin-layer Chromatography

The chromatoplates (20 x 20 cm) spread with 250 mμ of silica gel G were activated at 120 C for 1 hr. Two solvent systems were used: I, glacial acetic acid-cyclohexane-ethylacetate (3:7:23 v/v) (16), for the separation of free bile acids; and II, water-n-propanol-propionic acid-amylacetate (mixture of iso- and normal-amylacetate) 5:10:15:20 v/v) (17), for the sep-

TABLE II

Color Reaction to Anisaldehyde Reagent of Known Bile Acids and Bile Extracts on TLC Plates[a]

Color of bile acid standards		Color of unknown compounds	
Cholesterol	Purple	Band I	Greenish-purple
Lithocholic acid	Bluish-purple	Band II	Purple
Deoxycholic acid	Dark red	Band III	Bluish-purple
7-ketolithocholic acid	Yellow	---	---
Hyodeoxycholic acid	Greenish-blue	---	---
Chenodeoxycholic acid	Blue	---	---
Dehydrocholic acid	Red	Band IV	Purple
Glycolitholcholic acid	Purple	---	---
Cholic acid	Bluish-purple	Band V	Purple
Glycochenodeoxycholic acid	Purple	---	---
Glycodeoxycholic acid	Purple	Band VI	Purple
Glycocholic acid	Blue	---	---
Taurolithocholic acid	Purple	---	---
Taurochenodeoxycholic acid	Purple	Band VII	Purple
Taurocholic acid	Blue	Band VIII	Purple

[a]The developing solvent was water-propanol-propanol-propionic acid-amylacetate (5:10:15:20 v/v) for the separation of conjugated bile acids, and glacial acetic acid-cyclohexane-ethylacetate (3:7:23 v/v) for the separation of free bile acids. After spraying, the plates were heated at 110 C for 30-45 min. The bile-acid standards (except 7-ketolithocholic acid, KLC) were obtained from California Corporation for Biochemical Research, Los Angeles, California. KLC was obtained from Mann Research Laboratories, Inc., New York, N.Y. Cholesterol was recrystalized from petroleum ether, and ethanol in this laboratory. The unknown was a pooled bile extract from animals fed the basal diet of Table I.

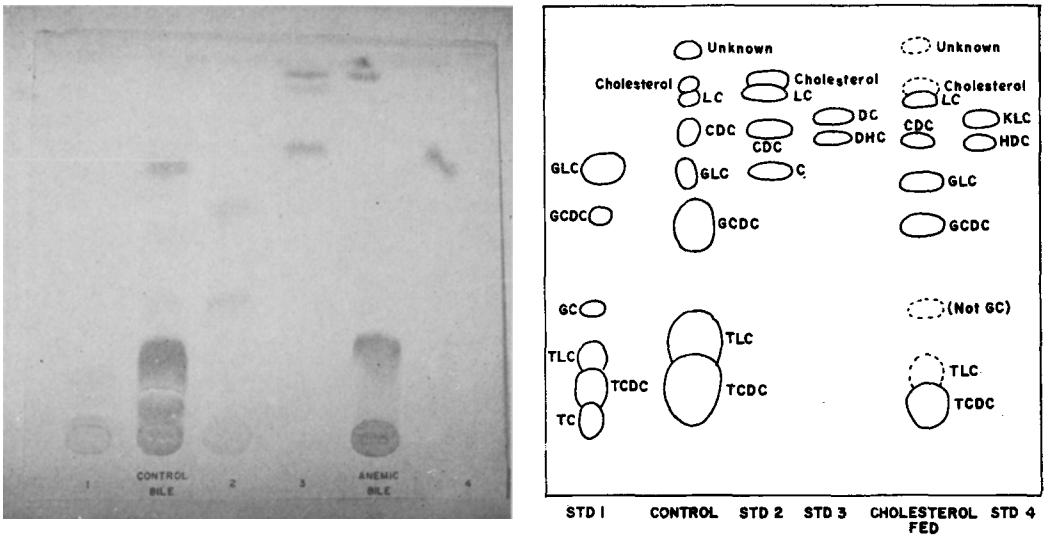

FIG. 1. Separation of bile acids from control and cholesterol-fed anemic guinea pigs. Silica gel G (0.25 mm); solvent II water-n-propanol-propionic acid-amylacetate (5:10:15:20 v/v); spray reagent: anisaldehyde 0.5 ml, conc. sulfuric acid 1 ml, acetic acid 50 ml; running time 90 min.; color developed by heating at 110 C for 30-45 min.; standard solutions 1-4 were mixtures of authentic samples, as indicated on the diagram, containing about 50 μg of each component. Control and cholesterol-fed samples were extracts of gallbladder bile from a guinea pig fed the basal diet (Table I) and one fed the same diet containing 1% cholesterol for nine weeks, respectively. The spot with Rf similar to GLY-C was probably a bile pigment. It was blue before spraying, and did not give glycine nor cholic acid on hydrolysis. GLY-CDC was contaminated with small amounts of GLY-DC and could not be separated in either solvent system. CDC did not contain KLC nor HDC. The amount of the control sample was too large, leading to an enlarged spot and an enhanced Rf value for TAURO-LC. However, rechromatography in solvent system I before and after hydrolysis showed it to contain TAURO-LC and LC respectively as the only components. Methods used for the identification of individual spots are described in the text.

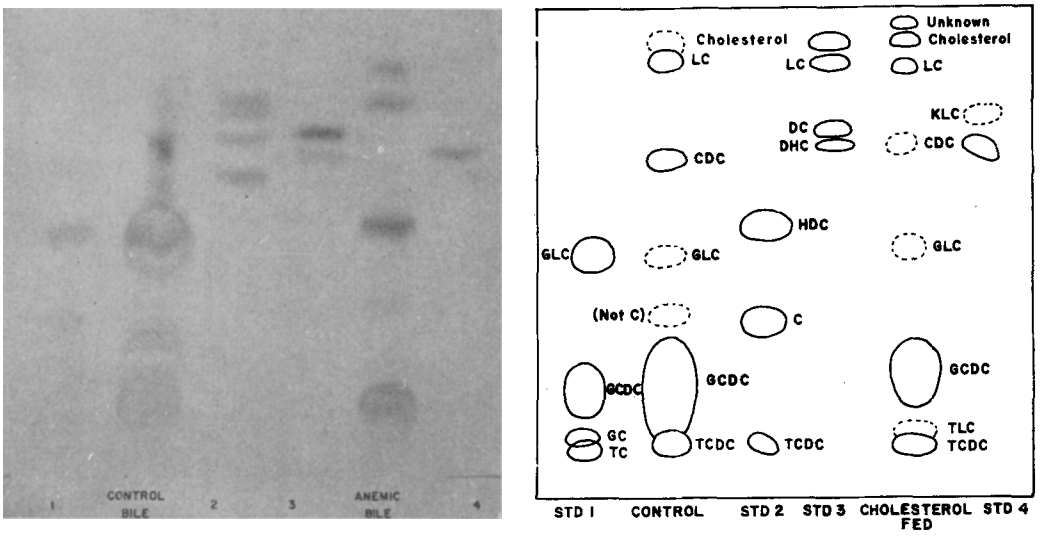

FIG. 2. Separation of bile acids from control and cholesterol-fed anemic guinea pigs. Silica gel G (0.25 mm); solvent I; glacial acetic acid-cyclohexane-ethylacetate (3:7:23 v/v); spray reagent anisaldehyde 0.5 ml, conc. sulfuric acid 1 ml, acetic acid 50 ml; running time, 30 min; color developed by heating at 110 C for 30-45 min. The spot with Rf similar to C was probably a bile pigment. It was not C because it was blue before spraying with anisaldehyde. For other explanations, see legend to Figure 1.

TABLE III

Comparison of Two Methods for the Quantitative Estimation of Gall-Bladder Bile Acids[a, b]

Animal number	Per cent of bile acid											
	GLY-CDC		TAURO-CDC		GLY-CDC/ TAURO-CDC		CDC		GLY-LC		TAURO-LC	
	PM	L-B	PM	L-B	PM	L B	PM	L-B	PM	L-B	PM	U
Control												
152	79.4	73.7	11.7	13.8	6.8	5.4	2.5	1.6	3.6	3.5	2.8	7.4
221+225	79.5	77.9	14.5	11.3	5.5	6.9	1.0	1.5	1.1	2.3	4.0	6.4
Cholesterol-fed												
214	50.1	56.5	41.0	35.2	1.2	1.6	0.5	1.3	2.9	1.4	5.4	3.7
232	64.4	64.1	30.3	25.8	2.1	2.5	0.7	1.6	0.8	1.7	3.9	3.8
255	69.6	62.2	23.5	22.5	3.0	2.8	0.1	1.4	0.9	4.7	5.7	9.3
204	67.7	64.4	27.7	26.5	2.4	2.4	0.2	1.4	0.5	2.0	3.7	4.8

[a]Aliquots of bile extracts were separated by TLC and estimated by the phosphomolybdic acid color reaction (PM) and the Usui reaction (U) for monohydroxycholanic acids or a modified Liebermann-Burchard reaction (L-B) for dihydroxycholanic acids. Control animals were fed the basal diet (Table I), cholesterol-fed animals were fed the same diets with the addition of 1% cholesterol for 10-17 weeks.

[b]CDC, chenodeoxycholic acid; LC, lithocholic acid; GLY-CDC and GLY-LC, glycochenodeoxy and -lithocholic acid; TAURO-CDC and TAURO-LC, taurochenodeoxy and -lithocholic acid.

aration of conjugated bile acids. The separated zones were located with: 10% phosphomolybdate solution in 95% ethanol (18); anisaldehyde (18); and iodine vapor (19). Plates which had been sprayed with phosphomolybdate were heated at 110 C for 5 to 10 min. The bile-acid spots turned dark blue against a yellow background. Anisaldehyde produced different colors with different bile acids. (See Table II)

Qualitative Identification of Bile Acids

The following procedures were used for identification of the bile acids: (a) The Rf values of the unknown samples were compared with those of bile-acid standards. (b) The color of unknown spots was compared with that of standards after spraying with anisaldehyde. (c) Intensification of the phosphomolybdate color reaction of an unknown spot when a known bile acid had been added to the sample before chromatography provided further evidence for the identity of the compound. (d) After separation by the II system, each band was scraped off the plate, extracted with methanol, and rechromatographed in the I system. Procedures (a) and (b) were then followed. (e) After separation by the II system, each band was scraped off the plate, extracted with methanol, and saponified with sodium hydroxide (20). The liberated free bile acids were then rechromatographed in either the I or II system, and identified as indicated under (a) to (d) above. (f) The spectra of the sulfuric acid solution of bile acids were examined. After TLC separation each

TABLE IV

Bile Acid Composition of Gallstones from Cholesterol-fed, Anemic Guinea Pigs[a]

Per cent of bile solids		Per cent of bile acids						
Bile acids	Cholesterol	BA/C	GLY-LC	TAURO-LC	GLY-CDC	TAURO-CDC	GLY-CDC/ TAURO-CDC	Bile solids, mg
17.6 ±2.99	1.4 +0.29	8.3 ±2.5	4.5 ±0.4	3.9 ±1.4	77.9 ±1.8	12.6 ±3.3	7.8 ±2.06	2.05 ±0.68

[a]Animals had been fed the basal diet (Table I) with the addition of 1% cholesterol for 10-17 weeks. Numbers are means ± SE of results from four animals. BA/C is the ratio of bile acids to cholesterol.

TABLE V

Rf Values of Bile-Acid Standards and Guinea Pig Bile-Extract Components[a]

Standard bile acids	Developing solvent II	Developing solvent I	Unknown	Developing solvent II	Developing solvent I	Identified as
Cholesterol	0.83	0.96	Band I	0.92	0.99	Unknown
Lithocholic acid (LC)	0.78	0.93	Band II	0.82	0.96	Cholesterol
Deoxycholic acid (DC)	0.77	0.84	Band III	0.79	0.93	LC
7-ketolitholcholic acid (KLC)	0.77	0.85	---			---
Hyodeoxycholic acid (HDC)	0.74	0.70	---			---
Chenodeoxycholic acid (CDC)	0.74	0.80	Band IV	0.76	0.76	CDC
Dehydrocholic acid (DHC)	0.72	0.79	---			---
Glycolithocholic acid (GLY-LC)	0.69	0.66	Band V	0.69	0.66	GLY-LC
Cholic acid (C)	0.66	0.48	b	0.48	0.46	---
Glycochenodeoxycholic acid (GLY-CDC)	0.59	0.25	Band VI	0.59	0.27	GLY-CDC+ GLY-DC
Glycodeoxycholic acid (GLY-DC)	0.59	0.25	---			---
Glycocholic acid (GLY-C)	0.43	0.04	c	0.45	0.45	---
Taurolithocholic acid (TAURO-LC)	0.28	0	Band VII	0.28	0	TAURO-LC
Taurochenodeoxycholic acid (TAURO-CDC)	0.20	0	Band VIII	0.18	0	TAURO-CDC
Taurocholic acid (TAURO-C)	0.09	0	---			---

[a]The Rf values presented came from a single plate. The numerical values varied from plate to plate, but the relationship of Rf values between unknowns and that of the standards remained the same. The developing system for the separation of conjugated bile acids was water-n-propanol-propionic acid-amylacetate (5:10:15:20 v/v) (II); glacial acetic acid-cycohexane-ethylacetate (3:7:23 v/v).Cholesterol was recrystalized from petroleum ether and ethanol in this laboratory. The unknown was a pooled bile extract from animals fed the basal diet of Table I. The bile-acid standards (except KLC) were obtained from California Corporation of Biochemical Research, Los Angeles, California; KLC was obtained from Mann Research Laboratories, Inc., New York, N.Y.

[b]A small spot of similar Rf in both solvent systems. It was not cholic acid but probably a bile pigment, because it was blue before spraying with anisaldehyde.

[c]A small spot of similar Rf in both solvent systems. It was not glyco-cholic acid but probably a bile pigment because it was blue before spraying and did not yield glycine nor cholic acid on hydrolisis..

zone from either a II or a I plate of bile extract was scraped off and dissolved in concentrated sulfuric acid. After the silica gel G was removed by centrifugation, the optical densities were read from 250 mμ to 500 mμ, with a Beckman DU spectrophotometer, and compared with spectra obtained from authentic bile acid samples.

Quantitative Determination of Bile Acids

The separated bands of bile acids on chromatoplates were visualized with iodine vapor. After sublimation of the iodine, the separated bands were collected by scraping off the silica gel bands.

Monohydroxycholanic acids were determined by the ferric chloride reaction of Usui (20).

Dihydroxycholanic acids and cholesterol were determined by a slight modification of the method described by Frosch and Wagner (21) using the Liebermann-Burchard reaction. The silica gel containing the individual bile acid was treated with 3.0 ml of a freshly-prepared mixture of ethylacetate-sulfuric acid (15:1 v/v) for 15 min, then 2.0 ml acetic anhydride were added. The solution was centrifuged to remove the silica gel, and the optical density of the supernatant was read at 420 mμ. Time for the development of maximal absorptions was 25 min for chenodeoxycholic acid, 30 min for cholesterol, 40 min for taurochenodeoxycholic acid, and 45 min for glycochenodeoxycholic acid. A concentration series of each bile acid standard was chromatographed on the same plate as the unknown sample. Therefore, each bile acid had its standard curve on every plate.

In order to check the above methods, some samples were also analyzed by the use of the phosphomolybdate color reaction (17). The TLC plate was sprayed with a 10% solution of phosphomolybdate in 95% ethanol. The plate was dried in air for 15 min, then heated at 65 C for 20 min. The colored spots were scraped off, the color was extracted into methanol and separated from silica gel by centrifugation. The optical density was read at 740 mμ. When aliquots of the same sample were analyzed by two different methods, the results were similar. Examples of such comparisons are presented in Table III. The lower limit of assay varied somewhat with the individual bile acid and the

method used. Cholic acid, at a level of 10 μg, for instance, gave an OD reading of 0.053 by the phosphomolybdate method but only 0.026 by the Lieberman-Burchard method. All bile acids tested could, however, be determined when present at a level of 10-20 μg. This corresponds to a level of 1-3% of total bile acids.

RESULTS

The bile of control animals was clear and faintly yellow, while that of cholesterol-fed animals was cloudy, yellowish to dark brown, and sometimes contained stones and small debris. Some of the analyses were performed on bile alone, while some samples contained the gall bladder membrane and all contents, including the concrements. Separate analysis of these membrane-plus-concrements showed that their bile acid composition resembled that of filtered bile from control animals more than that from cholesterol-fed animals, and that their bile acid to cholesterol ratio was much lower than in bile from either group (Table IV). Since, however, the amount of solids in these extracts was only 5% of total bile solids no significant alterations resulted when the composition of bile was determined with or without membrane-plus-concrements (see footnote to Table VI). We have, therefore, averaged all data for samples analyzed with or without bladder membrane and gallstones.

TLC of the bile extracts produced eight bands, as shown in Figures 1 and 2. The Rf values of these bands in both solvent systems are listed in Table V. Results show that the gallbladder bile of normal and cholesterol-fed guinea pigs has a similar composition, containing the monohydroxy- and dihydroxycholanic acid-lithocholic (LC), chenodeoxycholic (CDC) and deoxycholic (DC) acids-both as the free acids and as their glycine and taurine conjugates. No cholic nor 7-ketolithocholic acids (KLC) were detected. Table VI presents the results of the quantitative determination of the bile acids. Bile acids and their conjugates represented about 35% of bile solids. The major constituents were glyco- and taurochenodeoxycholic acid (GLY-CDC and TAURO-CDC) which accounted for 88% of total bile acids. GLY-LC, TAURO-LC and free CDC, DC, LC and cholesterol were present in smaller amounts. The major difference between the control and the cholesterol-fed groups was a change in the conjugation pattern of chenodeoxycholic acid. The proportion of GLY-CDC was lower in the cholesterol-fed animals than in controls, while that of TAURO-CDC was greater. This led to ratios of GLY-CDC to

TABLE VI

Bile-Acid Composition of Gallbladder Bile From Control and Cholesterol-Fed, Anemic Guinea Pigs[a]

	Per cent of bile solids		Per cent of bile acids								
	Bile acids	Cholesterol[b]	BA/C[c]	CDC	LC	GLY-LC	TAURO-LC	GLY-CDC[c]	TAURO-CDC[c]	GLY-CDC/TAURO-CDC[c]	Bile solids, mg
Control	35.1±4.1 (10)	0.5±0.1 (7)	70.2	1.4±0.3 (11)	1.5±0.3 (7)	3.8±0.6 (12)	7.0±1.0 (12)	74.3±1.6 (12)	12.9±1.5 (12)	6.4±0.8	36.9 (12)
Cholesterol-fed	33.0±9.9 (13)	1.4±0.2 (10)	23.6	1.6±0.2 (10)	1.5±0.2 (3)	3.4±0.6 (12)	7.7±1.2 (12)	60.6±2.2 (13)	27.2±2.1 (13)	2.4±0.3[d]	40.9 (13)

[a]Control animals were fed the basal diet (Table I), cholesterol-fed animals were fed the same diet with the addition of 1% cholesterol for 10-17 weeks. Numbers are means ± SE of the number of individual animals indicated by numbers in brackets. BA/C is the ratio of bile acids to cholesterol.

[b]The differences between the two diet groups was statistically different at $p < 0.05$.

[c]The difference between the two diet groups was statistically different at $p < 0.01$.

[d]This ratio had a mean of 2.38 and 2.43 for seven samples of filtered bile and six samples containing also membranes and concrements, respectively. GLY-CDC contained a small amount of GLY-DC.

TAURO-CDC of 6.4 and 2.4 in the control and cholesterol-fed animals, respectively. The cholesterol content was higher in the cholesterol-fed, anemic group than in controls. Since the amount of bile acids did not change significantly, the ratio of bile acids to cholesterol was considerably lower in the former than in the latter group.

DISCUSSION

The presence of biliary concrements in the cholesterol-fed, anemic guinea pigs was an incidental, but not unexpected observation in the present study. The induction of gallstone formation by dietary cholesterol in guinea pigs had previously been reported (30). Gallstones found in mice fed cholesterol and cholic acid contained over 90% cholesterol (31), while those of guinea pigs contained only small amounts of cholesterol (Table IV). The main components of these calculi were bile salts and, presumably, calcium salts of bilirubin and other bile pigments, breakdown products of hemoglobin. It has been reported that gallstones with a core of such substances are frequently observed in hemolytic anemias (32,33) and may, therefore, be a consequence of the anemia and unrelated to the metabolism of cholesterol.

Studies of the mechanism of gallstone formation using hamsters fed a fat-free diet (34) or guinea pigs fed a diet containing cholestyramine (35) showed that the ratio of bile acid to cholesterol in bile of stoneformers was much lower than in controls. A similar effect has been observed in the present investigation (Table VI).

Most previous reports on the bile acid composition of gall bladder bile from guinea pigs have shown, as we do, that chenodeoxycholic acid is the major component and that about 80% of it is conjugated with glycine (22-26). Peric-Golia and Jones (27,28) have reported the isolation of cholic acid from guinea pig bile and found it conjugated exclusively with taurine. These divergent results may be due to differences between strains of animals and experimental conditions. The composition of diet and the thyroid state of the animals, for instance, markedly affect the type and quantity of bile acids in man and animal (29).

The ratio of glycocholanic to taurocholanic acids present in bile of normal animals differs widely in different species. Kritchevesky has pointed out (36) that in those mammalian species resistant to cholesterol-induced atherosclerosis (rat, dog, cat), the bile acids are conjugated almost exclusively with taurine. The susceptible species, on the other hand (rabbit, pig, monkey, man), have both types of conjugates, with glycocholanic acids greatly in excess in rabbit and pig and less so in man and monkey. The normal guinea pig appears to fall into the group of susceptible species with a high ratio of glyco-chenodeoxy cholic to tauro-chenodeoxy cholic acid. The susceptibility to injury by cholesterol in the guinea pig leads, however, not to atherosclerosis, but to anemia.

Changes in the conjugation pattern of bile acids leading to a greater proportion of taurine conjugation have been reported to occur under certain circumstances. Ekdahl (37) reported that homogenates of human livers under certain pathological conditions (obstructive jaundice of more than 10 days' duration, or cirrhosis) contained a higher proportion of cholic acid conjugated with taurine than normally. In his studies of human subjects, cholesterol-fed, anemic guinea pigs are enlarged, fatty, and show striking pathological changes (14). The change of conjugation pattern which we have observed may, therefore, be due in part to changes in liver function.

Hellstrom and Sjovall (39) reported that the ratio of glycine to taurine conjugates was high (7.2-14.5) in hypothyroid patients. After treatment, the ratio returned toward the normal level (around 3), suggesting that the metabolic pathway to taurine conjugated bile acids is in some way influenced by thyroid hormones. Failey (40) observed an increase of biliary taurodeoxycholic acid in man after an oral dose of ^{14}C-cholesterol. He suggested that taurine conjugation may be relatively more efficient than glycine as a means of hepatic excretion of bile acids. The increased proportion of taurine conjugates in the cholesterol-fed guinea pig may, therefore, be in part a compensatory mechanism to excrete the increased load of cholesterol.

A third possible mechanism for the decrease of glycine-conjugates in the cholesterol-fed guinea pig may be related to the effect of the anemia on the availability of glycine. The red blood cells of cholesterol-fed anemic guinea pigs had a life span of 4-19 days, which was very much shorter than that of control guinea pigs, 60 days (8). The high rate of hemoglobin formation in these animals may, therefore, exceed their capacity for the formation of glycine.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service Grant AM-08480. Care and autopsy of the animals and technical assistance in the analytical work was performed by W. Yamanaka.

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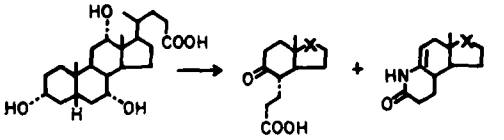
[Received July 30, 1968]

SHORT COMMUNICATIONS

Novel Microbiological Degradation Products From Cholic Acid

We have recently reported on the microbiological conversion of cholic acid (I) into 4α-(2-carboxyethyl)-5-oxo-7aβ,γ(*R*)-dimethyl-3aα-hexahydroindan-1β-butyric acid (IIa) with *Arthrobacter simplex* (1) and also on the formation of the amino acid conjugates of the acid IIa with *Corynebacterium equi* (2). In continuing our studies on the microbiological degradation of bile acids, we have found that *Streptomyces rubescens* (3), cultured in a medium containing cholic acid (I) as the sole source of carbon, produces unexpected products having an enamine lactam structure (III).



I II III

a. X = CH·CH(CH$_3$)·CH$_2$·CH$_2$·COOH
b. X = CH·CH(CH$_3$)·CH$_2$·CH$_2$·CONH$_2$
c. X = CH·CH(CH$_3$)·CO·CH$_3$
d. X = CH·CH(CH$_3$)·COOH
e. X = C:O

The exposure of cholic acid (I) to *S. rubescens* resulted in the formation of the following products after a disappearance of some metabolites reported previously (3) from the incubation mixture: The acid IIa, 1β-(1(*R*)-methyl-3-carbamoylpropyl)-5-oxo-7aβ-methyl-3aα-hexahydroindan-4α-propionic acid (IIb), 3-oxo-6aβ,γ(*R*)-dimethyl-2,3,4,6,6aβ,7,8,9,9aα,9bβ-decahydro-1*H*-cyclopenta[*f*]quinoline-7β-butyric acid (IIIa), 3-oxo-6aβ,γ(*R*)-dimethyl-2,3,4,6,6aβ,7,8,9,9aα,9bβ-decahydro-1*H*-cyclopenta[*f*]quinoline-7β-butyramide (IIIb), 6aβ-methyl-7β-(1(*S*)-acetylethyl)-2,3,4,6,6aβ,7,8,9,9aα,9bβ-decahydro-1*H*-cyclopenta[*f*]quinolin-3-one (IIIc), 3-oxo-6aβ,α(*S*)-dimethyl-2,3,4,6,6aβ,7,8,9,9aα,9bβ-decahydro-1*H*-cyclopenta[*f*]quinoline-7β-acetic acid (IIId), and 6aβ-methyl-2,3,4,6,6aβ,7,8,9,9aα,9bβ-decahydro-1*H*-cyclopenta[*f*]quinoline-3,7-dione (IIIe). All the structures of the nitrogen-containing products, except for the product IIIc isolated recently, were conclusively established by a partial synthesis. A detailed discussion of the isolation and characterization of these products, except for the product IIIc, will be published elsewhere (4) in the near future. The product IIIc, mp 242-242.5 C and [α]D + 122.1° in ethanol, was obtained by silicic acid chromatography (20% acetone in dichloromethane) of a mixture of degradation products and its constitution was deduced from the following spectrometric data: $\lambda_{max}^{alc.}$ 232.5 mμ (ε = 12,800); ν_{max}^{KBr} 3215 and 3075 (NH), 1713 (methyl ketone), and 1676 and 1669 cm^{-1} (enamine lactam); NMR (in $CDCl_3$) 0.75 (singlet, 3H, 6aβ-Me), 1.12 (doublet, J = 6.7 cps, 3H, secondary Me in the side chain), 2.11 (singlet, 3H, methyl ketone in the side chain), 4.79 (multiplet, 1H, vinylic 5-H), and 7.79 ppm (broad-singlet, 1H, lactam 4-H). The product IIIc, however, may be an artifact produced in the isolation process through a decarboxylation of the β-oxocarboxylic acid, 3,β-dioxo-6aβ,γ(*S*)-dimethyl-2,3,4,6,6aβ,7,8,9,9aα,9bβ-decahydro-1*H*-cyclopenta[*f*]quinoline-7β-butyric acid, derived from the product IIIa in a manner analogous to the fatty acid β-oxidation mechanism. *S. rubescens* was not able to utilize the product IIIe as the sole carbon source at a significant rate. However, it was found that this organism was capable of utilizing 1,5-dioxo-7aβ-methyl-3aα-hexahydroindan-4α-propionic acid (IIe) as the sole carbon source and that one of the metabolites of the compound IIe with this organism was the product IIIe.

It is not yet confirmed whether the overall reactions involved in the enamine lactam formation are enzymic or not. However, the precursors of the enamine lactams (III) may be the corresponding δ-oxocarboxylic acids (II), as suggested from a conversion of the compound IIe into the product IIIe, and an enzymic amidation of the propionic acid side chain in the compound II molecule is probably an early step in the reaction sequence of the enamine lactam formation. A similar amidation of the butyric acid side chain in the acid IIa molecule perhaps produces the product IIb. The compound IIe and its reduction product have been known as significant intermediates in the microbiological degradation sequence of some steroids (5-7). The present experiment demonstrates that the compound IIe also may be a key intermediate in the degradative pathway of cholic acid (I). Con-

sidering the chemical structures of the degradation products, a possible pathway for the cholic acid degradation by *S. rubescens* may be

I → → (IIa ⇌ IIb ⇌ IIIb) and/or (IIa ⇌ IIIa ⇌ IIIb) → (IId ⇌ IIId) → (IIe ⇌ IIIe) → further degradation.

SHOHEI HAYAKAWA
SHIGERU HASHIMOTO
TADAMASA ONAKA[1]
Shionogi Research Laboratory
Shionogi & Co., Ltd.
Fukushima-ku, Osaka
Japan

[1]Present address: Research Foundation, Itsuu Laboratory, Tokyo, Japan.

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[Received October 16, 1968]

Mass Spectra of α,ω-Diiodoalkanes

The αω-diiodoalkanes have several important applications in lipid chemistry. The ether terminal fragments of unsaturated alkyl glycerols yield α,ω-diiodoalkanes when the alkyl glycerols are cleaved by reductive ozonolysis with lithium aluminum hydride and the products are reacted with hydriodic acid. These diiodide derivatives are used for the location of olefinic bonds in alkyl glycerols (1). The same reaction sequence yields α,ω-diiodoalkanes from the carboxyl terminal fragments of the methyl esters of unsaturated fatty acids and these diiodides may be used in place of aldehydoesters and dicarboxylic acids in cleavage studies with fatty acids. Plant waxes contain a number of long-chain fatty acids with ω-hydroxy groups (2, 3) and medium-chain fatty acids are converted to ω-hydroxy fatty acids by bacterial (4) and mammalian (5, 6) enzyme complexes. These ω-hydroxy acids may be identified after their reduction to diols with lithium aluminum hydride and conversion to diiodides with hydriodic acid. Furthermore, the α,ω-diols in the neutral estolide fractions of plant waxes (3) may be identified as their diiodide derivatives.

The α,ω-diiodoalkanes are readily separated by gas-liquid chromatography (GLC) (1). In the present study, we show that the mass spectra of the diiodides exhibit several characteristic features which facilitate their identification. The combination of GLC and mass spectrometry provides a means for the rapid separation and identification of microgram quantities of these derivatives.

Materials included 1,3-diiodopropane and 1,5-diiodopentane (Eastman Organic Chemicals, Rochester, N. Y.); 1,4-diiodobutane (Aldrich Chemical Co., Milwaukee, Wis.); 1,6-diiodohexane and 1,9-diiodononane synthesized from the corresponding diols (Matheson Coleman and Bell, Cincinnati, Ohio) by refluxing with hydriodic acid (1); 1,10-diiododecane and 1,11-diiodoundecane synthesized from diols

TABLE I
Characteristic Ions in Mass Spectra of α,ω-Diiodoalkanes

	I-(CH₂)ₙ-I						
Ion	3	4	5	6	9	10	11
	m/e[a]						
M^+	296(35)	310(11)	324(36)	338(54)	380(24)	394(27)	408(16)
M^+-I	169(69)	183(100)	197(88)	211(73)	253(22)	267(29)	281(20)
$M^+-(I+HI)$	41(76)	55(94)	69(98)	83(83)	125(17)	139(10)	153(5)
HI^+	128(100)	128(35)	128(40)	128(39)	128(34)	128(37)	128(21)
I^+	127(53)	127(19)	127(21)	127(21)	127(18)	127(19)	127(11)
I^+_2	254(8)	254(5)	254(4)	254(2)	< 0.3	< 0.3	< 0.3
Base peak	128	183	41	55	55	55	55

[a]Per cent of base peak in parenthesis.

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SHOHEI HAYAKAWA
SHIGERU HASHIMOTO
TADAMASA ONAKA[1]
Shionogi Research Laboratory
Shionogi & Co., Ltd.
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Japan

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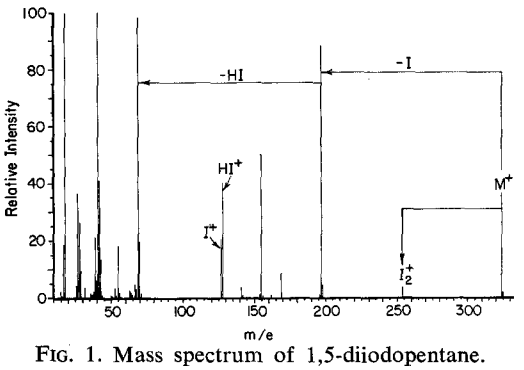

FIG. 1. Mass spectrum of 1,5-diiodopentane.

prepared from methyl undecenoate (Eastman Organic Chemicals, Rochester, New York) and methyl *cis*-vaccenate (Hormel Institute, Austin, Minn.) by reductive ozonolysis with lithium aluminum hydride. Diiodide purity was established by GLC (1).

The mass spectral data were obtained using an A.E.I. MS-9 mass spectrometer. High resolution data were obtained by an on-line digitization system with subsequent computer-processing of the digital tape. Most of the mass spectral data were obtained using the direct introduction sample probe with a source temperature of 150 to 200 C and an ionizing voltage of 70 eV. A mass spectrum obtained by introducing the 1,6-diiodohexane into the mass spectrometer through the heated inlet (all glass except for the valves) showed molecular ion peaks corresponding to 1,6-bromoiodohexane and 1,6-chloroiodohexane. These peaks were absent in the mass spectrum of the 1,6-diiodohexane introduced through the direct probe. Consequently, a halogen exchange must have occurred in the heated inlet system which is normally maintained at 200 C. The source of the chloride and bromide is not known.

The mass spectra of each of the diiodides examined in this study exhibited prominent peaks corresponding to the molecular ion (M^+), M^+-I, $M^+-(I + HI)$, HI^+, I^+, and a series of $I(CH_2)_n^+$ ions as well as hydrocarbon ions which generally corresponded to the series $C_nH_{2n-1}^+$ and $C_nH_{2n-3}^+$. The mass spectrum of 1,5-diiodopentane (Fig. 1) illustrates these features.

Molecular Ions (M^+): All of the α,ω-diiodoalkanes gave moderately strong molecular ion peaks (Table I). The intensities of the molecular ions were unexpected in view of the very small molecular ion peaks reported (7) for monoiodoalkanes containing more than six carbon atoms. However, this difference is partly due to the different instruments and

TABLE II

Relative Intensities of $I(CH_2)^+_n$ Ions Obtained in the Fragmentation of α,ω-Diiodoalkanes

	I–$(CH_2)_n$–I						
$I(CH_2)^+_n$	3	4	5	6	9	10	11
	Per cent of Base Peak						
1	3	4	5	2	2	1	1
2	6	16	50	31	36	42	33
3	54	0.5	9	20	22	29	23
4		100	<1	5	24	31	26
5			88	0.5	22	24	18
6				36	12	21	14
7					0.4	10	12
8					0.5	0.5	5
9					22	<1	1
10						29	<1
11							20

inlet systems used. The mass spectrum of 1-iododecane obtained using the direct probe sample introduction technique showed a molecular ion with an intensity 3% of the base peak (m/e 43). No molecular ion was detected in the previously reported (7) mass spectrum of 1-iododecane. The inlet temperature is known to affect the relative intensity of the molecular ion (8).

M^+-I and $M^+-(I + HI)$ Ions: Identification of the molecular ion is facilitated by the occurrence in each case of an equally strong or stronger peak at 127 mu (mass units) below the molecular ion corresponding to the loss of an I atom. The M^+-I ion subsequently undergoes loss of HI to give a prominent peak at 255 mu below the molecular ion. Both of these fragmentations are accompanied by unusually large metastable ion peaks. Loss of HI followed by I or consecutive loss of two I atoms are negligible fragmentations pathways.

I^+, HI^+ and I_2^+ Ions: The ratio of intensities of I^+ to HI^+ ions (127-128) is remarkably constant, corresponding to 0.53 ± 0.02 for each of the diiodides examined.

Formation of the I_2^+ ion (m/e 254) is observed in the lower members of the series. Its relative intensity decreases with increasing chain length and becomes of negligible intensity in the C-9 and larger diiodides.

$I(CH_2)_n^+$ Ions: This series of ions accounts for a substantial proportion of the total ion current. Their relative intensities within each spectrum form a characteristic pattern (Table II). In all cases, the ICH_2^+ ion peak (m/e 141) is of low intensity. The $IC_2H_4^+$ ion peak (m/e 155), however, is one of the stronger peaks in each spectrum. The remaining $I(CH_2)_n^+$ peaks decrease in relative intensity until reaching the ion corresponding to M^+-I

which, as previously described, is always intense.

RODGER L. FOLTZ
S. RAMACHANDRAN
DAVID G. CORNWELL
The Battelle Memorial Institute
and The Department of
Physiological Chemistry
The Ohio State University
Columbus, Ohio 43210

ACKNOWLEDGMENT

This study was supported in part by Research Grant GM 09506 from the National Institutes of Health, United States Public Health Service.

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In Vitro Biosynthesis of β-Carotene by Bovine Corpus Luteum Tissue

In 1913, Willstätter (1) discovered β-carotene in the bovine corpus luteum. Its high concentration (2), the close relationship of carotenoid and steroid synthesis (3,4), and Porter's isolation of geranylgeranyl pyrophosphate synthetase from pig liver (5) caused us to question the assumption that only plant tissues could synthesize carotenoids (6). This report presents data demonstrating the ability of bovine corpus luteum tissue to synthesize β-carotene.

Preparations, either slices prepared with a Stadie hand microtome or homogenates made with an all glass hand homogenizer, were made from corpora lutea of freshly slaughtered cows and incubated at 37.5 C with ^{14}C-sodium acetate (New England Nuclear). The radioactive sodium acetate was prepurified by paper chromatography. After incubation, the tissue was homogenized with peroxide-free ether (distilled over ferrous chloride). After the ether was evaporated with a stream of nitrogen, each sample was dissolved in absolute ethanol-60% potassium hydroxide (10:1 v/v) and saponified overnight in the dark at room temperature under nitrogen. Water was added and the nonsaponifiable material extracted with ether and washed with water to remove alkali (7). The ether was evaporated with a stream of nitrogen. The nonsaponifiable fractions were dissolved in acetone, and 10% of each sample was spotted on the corner of a 250 μ thick, 20 x 20 cm silica gel G thin layer chromatography (TLC) plate. In the case of the blank, standard β-carotene was added to the spot. The plates were developed in two dimensions with benzene-petroleum ether (6:4) and acetone-petroleum ether (1:9). Following development, the spot corresponding to β-carotene standards was scraped off and eluted with acetone, using a modified Swinny adapter (8), into liquid scintillation vials. After evaporation of acetone, β-carotene was dissolved in 1 ml of a 10% benzoyl peroxide-toluene solution and placed in the sunlight to bleach the yellow color which can quench in liquid scintillation counting (9). Phosphor solution (10 ml of a solution of 9 g PBD + 50 mg POPOP/liter of toluene) was added and the samples were counted in a Packard TriCarb model 3310 liquid scintillation spectrometer.

Four incubations (Tables I-IV) were run. Three incubations were performed with isotonic vanadyl sulfate ($VOSO_4$) since Azarnoff et al. (10) reported that this compound inhibits squalene synthetase and it might, therefore, push the reaction toward β-carotene.

TABLE I
Incubation I[a]

Sample	CPM
Slices, Nitrogen	119
Slices, Air	71
Slices, 95% Oxygen-5% CO_2	89
Homogenate, Nitrogen	0
Homogenate, Air	3
Homogenate, 95% Oxygen-5% CO_2	0

[a] Each flask contained 5 ml isotonic buffer medium (0.12 M NaCl, 0.0047 M KCl, 0.0025 M $CaCl_2$, 0.0012 M KH_2PO_4, 0.0012 M $MgSO_4$, 0.0008 M $MnCl_2$, and 0.0247 M CO_2-saturated $NaHCO_3$), pH 7.4 and 430,000 CPM 1-^{14}C-sodium acetate (2 μc/μmole). The incubation was for 20 hr at 37.5 C.

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TABLE II
Incubation II[a]

Sample	State of tissue	Mg corpus luteum	μmoles $VOSO_4$	CPM/g
H-0	Homogenate	327.1	0	89
H-1	Homogenate	572.3	15.4	741
H-2	Homogenate	416.3	30.8	158
S-0	Slices	356.8	0	8720
S-1	Slices	329.7	15.4	12780
S-2	Slices	571.8	30.8	32750

[a] Each flask contained 5 ml isotonic buffer medium and 10^6 CPM 1-^{14}C-sodium acetate. The incubation was under nitrogen for 20 hr at 37.5 C.

Proof for the identification of β-carotene was as follows. The spot scraped off each plate was the most brightly colored of all the spots, and β-carotene is the predominant yellow pigment of the corpus luteum. The spot had the same R_f in both solvent systems as authentic β-carotene (0.74 benzene-petroleum ether and 0.79 in acetone-petroleum ether). When measured with a Cary model 14 spectrophotometer, the radioactive samples had the same spectrum in hexane (peaks at 477, 450 and 425 mμ) as the standard and as reported in the literature (11). Infrared spectra of potassium bromide disks of sample and standard, taken with a Perkin Elmer model 257 infrared spectrophotometer, matched each other. The most radioactive samples were pooled; added to non-radioactive β-carotene and recrystallized to constant specific activity from hexane-95% ethanol (5:1). After each recrystallization, the crystals were dissolved in 2-3 ml of benzene, and aliquots removed for counting and spectrophotometric measurement at 450 mμ in hexane. Using 2505 for the $E\ {}_{1\ cm}^{1\%}$ value (7), values of 41.1, 35.4, and 36.9 counts per minute per milligram were obtained after three recrystallizations, thus demonstrating that the radioactivity was in β-carotene.

It can be seen that β-carotene was synthesized in the bovine corpus luteum. An average recovery of 63% (range: 61-65%) was obtained for β-carotene (measured spectrophotometrically). A conversion of 1.8% of the ^{14}C-sodium acetate into β-carotene was found under our best conditions. Since the slices gave a better yield than homogenates, one could suspect a membrane phenomenon. More likely the many different reactions between acetate and β-carotene may take place in different organelles as is the case in steroid synthesis (12). Homogenization dilutes the intermediates into the total buffer volume rather than just the volume of the cellular cytoplasm.

TABLE III
Incubation III[a]

Sample	Mg corpus luteum slices	μmoles $VOSO_4$	CPM/g
0	310.6	0	9,300
2	303.0	30.8	36,400
4	674.6	61.6	50,800
6	241.0	92.4	6,460
8	512.7	123.2	4,420
10	443.6	154.0	10,100

[a] Each flask contained 5 ml buffer medium and 850,000 CPM 2-^{14}C-sodium acetate (2 μc/μmole). The incubation was under nitrogen for 8 hr at 37.5 C.

TABLE IV
Incubation IV[a]

Sample	Mg corpus luteum slices	μmoles $VOSO_4$	micrograms β-carotene	CPM/g
0	360.7	0	9.8	265
30	512.5	46.2	13.9	9180
35	337.1	53.9	4.2	21750
40	408.4	61.6	7.2	5380
45	372.5	69.3	5.7	100
50	287.7	77.0	5.5	0
80	396.5	123.2	6.5	0
100	417.4	154.0	4.0	119
120	477.4	184.8	3.2	9

[a] Each flask contained 5 ml buffer medium and 632,500 CPM 2-^{14}C-sodium acetate. The incubation was under nitrogen for 8 hr. The amount of β-carotene was measured spectrophotometrically (Beckman DU-2 spectrophotometer) between elution and bleaching at 450 mμ in spectral grade hexane.

Maximum β-carotene synthesis was reached at a $VOSO_4$ concentration of 350μ liter/5 ml buffer (i.e., 0.01 molar); slightly higher concentrations inhibited the conversion. At even higher concentrations, above 0.023 molar, the synthesis was once more increased. Both incubations III and IV show this same effect. This may be because $VOSO_4$ inhibits other enzymes than squalene synthetase (10,13,14), and that it may react with varying enzymes with differing inhibitor constants.

Nitrogen was the best atmosphere for the incubation, probably because the cyclization of squalene, in this case a side reaction, requires molecular oxygen (15). These experiments demonstrated the ability of bovine corpus luteum tissue to synthesize β-carotene.

BARRY M. AUSTERN[1]
ANTHONY M. GAWIENOWSKI
Department of Biochemistry
University of Massachusetts
Amherst, Massachusetts 01002

[1] NIH Predoctoral Trainee

ACKNOWLEDGMENT

Supported in part by funds from the U.S.D.H.E.W., National Institute of Child Health and Human Development Training Grant HD204.

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[Received December 2, 1968]

Mass Spectrometry of Derivatives of Cyclopentenyl Fatty Acids

Several fatty acids with a cyclopentenyl ring at the end of the fatty acid chain are found in the seed fats of *Hydnocarpus* and other genera of Flacourtiaceae which are used in treatment of leprosy. These acids include hydnocarpic (11-(cyclopent-2-enyl)undecanoic), chaulmoogric (13-(cyclopent-2-enyl)-tridecanoic) and gorlic (13-(cyclopent-2-enyl)-tridec-6-enoic) acids. This paper describes how the mass spectra of these and certain of their derivatives could aid in identification of these and similar substances.

Hydnocarpic, chaulmoogric and gorlic acids were obtained from *Hydnocarpus wightiana* seed oil, and were at least 99.5% pure by GLC and TLC. The saturated derivatives were prepared either by hydrogenation over palladium oxide catalyst or by deuteration with deuterohydrazine ($N_2D_4 \cdot D_2O$; Merck, Sharpe & Dohme of Canada, Ltd.) in anhydrous dioxane. Methyl hydnocarpate was reduced to the alcohol with lithium aluminum hydride, and the methanesulfonate derived from this was reduced similarly to the hydrocarbon.

The mass spectra were recorded with a Hitachi Perkin-Elmer RMU-6D instrument using the liquid injection system at 160 C. Spectra were obtained at an ionization potential of 80 eV and also at the lowest voltage which gave a countable spectrum for the following: methyl hydnocarpate, ethyl hydnocarpate, methyl hydnocarpate-cyclopropane derivative, hydnocarpic acid, methyl dihydrohydnocarpate, methyl dideuterohydnocarpate, dihydrohydnocarpic acid, 1-undecylcyclopent-2-ene, 1-undecylcyclopentane, 1-decyclohexane, methyl chaulmoograte, methyl dihydrochaulmoograte and methyl gorlate.

The mass spectrum of methyl hydnocarpate at 80 eV is depicted in Figure 1. Prominent peaks are the molecular peak (M = 266), m/e = 185 (cleavage β to the cyclopentene ring), m/e = 153, ($CH_2 = CH(CH_2)_7CO-$), and m/e = 82. This last, the base peak at low voltages, presumably represents a fragment comprising the cyclopentene ring plus one other methylene group and a hydrogen atom transferred from the chain. At 80 eV, the major peak is at m/e = 67, probably the cyclopentene ring itself. These assignments are made because although in the spectrum of the ethyl ester, those peaks at m/e = M-32 and m/e = 185 are replaced by similar ones at m/e = M-46 and m/e = 199, those at m/e = 153 and m/e = 82 remain unchanged. The McLafferty rearrangement ion (1) at m/e = 74 is apparent though not prominent. Many of these peaks were even more pronounced in the low voltage spectrum. In 1-undecylcyclopent-2-ene the base peak was also at m/e = 82, though the presence of a very large peak at m/e = 67 indicates that fragmentation adjacent to the cyclopentene ring also occurs in this case. Cleavage also occurs in the aliphatic chain and smaller peaks are found at m/e = 194 (M-28), m/e = 180 (M-43) and m/e = 166 (M-56).

The spectra of methyl chaulmoograte were analogous to those of methyl hydnocarpate. Prominent metastable peaks confirmed the following transitions: m/e = 233.5, 294 → 262 + 32; m/e = 154.3, 294 → 213 + 81; m/e = 153.8, 213 → 181 + 32; m/e = 146.8, 181 → 163 + 18.

The spectra of methyl gorlate (Fig. 1) exhibited quite different features and hydrocarbon peaks predominated at both high and low ioni-

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The spectra of methyl gorlate (Fig. 1) exhibited quite different features and hydrocarbon peaks predominated at both high and low ioni-

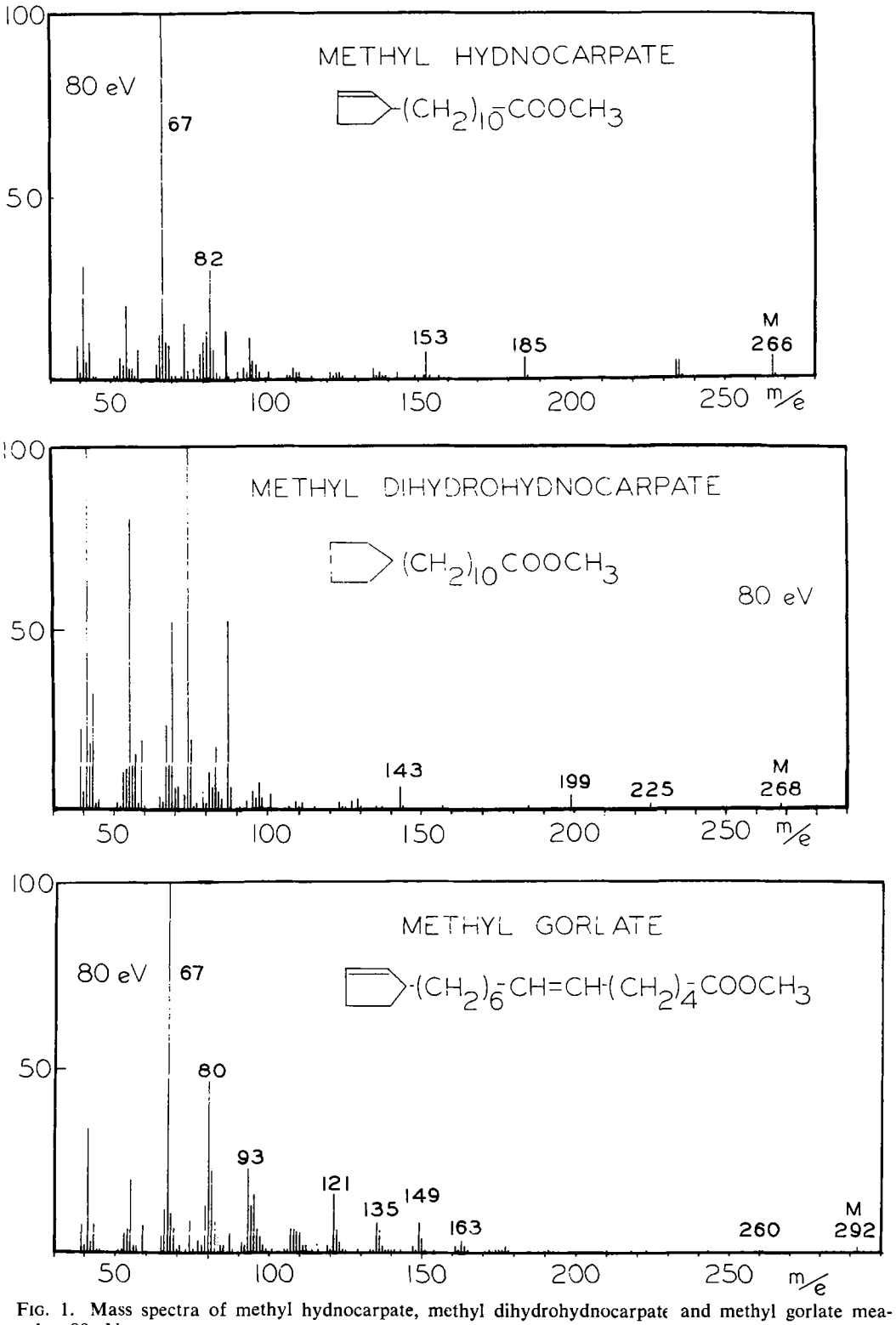

FIG. 1. Mass spectra of methyl hydnocarpate, methyl dihydrohydnocarpate and methyl gorlate measured at 80 eV.

zation potentials. In the 80 eV spectrum, the base peak was at m/e = 67, and the only other intense peak was m/e = 80. At low voltage the base peak was m/e = 80, formally equivalent to the dihydrofulvene ion $C_5H_6{=}CH_2{}^+$, and series of similar alkyl dihydrofulvene ions occurred, with the probable structures $C_5H_6{=}CH(CH_2)_n{}^+$ and $C_5H_6 = CH(CH_2)_nH^+$. The intense peaks were m/e = 149 (n = 5) and m/e = 121 (n = 3), with smaller ones at m/e = 135 (n = 4) > 178 (n = 7) > 163 (n = 6) > 94 (n = 1) > 108 (n = 2). The only oxygen-containing ions were the small molecular peak and a small peak at m/e = 260 formed by loss of methanol.

The mass spectra of methyl dihydrohydnocarpate (Fig. 1) were very similar to those of methyl palmitate, m/e = 74 being the base peak for both. In the high mass region, peaks at m/e = 225 (M-43), m/e = 199, and m/e = 143 occurred, which are also characteristic of normal fatty acid esters (2). At low voltages, though the base peak was still at m/e = 74, the peak at m/e = 199 ($M - C_5H_9$ring) was almost as large, and those at m/e = 143 and 225, characteristic of normal saturated methyl esters, were smaller. Fission to give a fragment $-(CH_2)_{10}CO_2CH_3$ occurs with most long straight-chain saturated fatty acids at high voltages, but virtually disappears at low energy. The presence of the large peak at m/e = 199 at a low voltage, therefore is in this case indicative of fragmentation at a cyclopentane ring. The spectra of the ester deuterated in the cyclopentane ring confirmed that fragmentation occurred in the manner described, for the only peaks obviously containing deuterium were the peak at M = 270 and that at m/e = M-43, the latter resulting from a rearrangement involving the expulsion of a 3 carbon fragment from the carboxyl end of the chain (3). Analogous spectra were given by methyl dihydrochaulmoograte, though an ion at m/e = 69 which might be expected for the cyclopentane ring itself, was not abundant. In contrast to this, the spectra of 1-undecylcyclopentane exhibited base peaks at m/e = 69 at both high and low voltages, corresponding to fragmentation at the cyclopentane ring. Fission also occurred at virtually every position in the aliphatic chain, however, to give regular series of ring-containing and aliphatic fragments. Similarly, 1-decylcyclohexane (supplied by American Petroleum Institute) had as its base peak at both high and low voltages an ion of m/e = 83, implying ready cleavage at the cyclohexane ring.

The mass spectrum of methyl 11-cyclohexylundecanoate isolated from butter fat (4) was reported to be so similar to that of the corresponding normal saturated acid at high voltage that the size of the ring could not be deduced. In our studies the mass spectra of cyclopentenyl and cyclopentyl fatty esters are distinct from those of corresponding normal unsaturated or saturated esters. Particularly helpful spectra were obtained at low ionization potentials, although such spectra are not quantitatively reproducible, and are therefore not shown here. With the cyclopentenyl esters, the principal mode of cleavage is β to the 5-membered ring, whereas with the corresponding saturated compounds, cleavage is adjacent to the ring. The additional double bond in methyl gorlate enhanced stability of cyclic ions which dominate its spectra. At 80 eV, the spectra of the saturated cyclic substances are so similar to those of the corresponding normal saturated esters that it was impossible to determine the size of the ring. At low ionization potentials, however, the distinction became obvious.

W. W. CHRISTIE[1]
D. REBELLO
R. T. HOLMAN
The Hormel Institute,
University of Minnesota
Austin, Minnesota 55912
and
University Department of Chemical Technology
Bombay, India

[1]Present address: Hannah Dairy Research Institute, Ayr, Scotland.

ACKNOWLEDGMENTS

This investigation was supported in part by PHS research grants HE 03559 and HE 08214 from the National Institutes of Health, Public Health Service, and The Hormel Foundation. H. Hayes operated the mass spectrometer and H. K. Mangold supplied the samples of methyl chaulmoograte and methyl gorlate.

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[Received November 29, 1968]

Changes in Phospholipid Composition of Human Aorta With Age

Changes in the amount of sphingomyelin in human aorta related to both age and atherosclerosis have been reported (1). The uncertainties with regard to the effects of postmortem changes, sampling procedures and analytical techniques in the earlier studies prompted us to reinvestigate the relationship of age and phospholipid distribution. With newer techniques for sampling and phospholipid analysis, we find that aorta sphingomyelin increases exponentially with age rather than linearly as previously reported. This exponential increase is accompanied by an exponential decrease of most of the other phospholipids.

We extracted total lipid with chloroform-methanol (2:1) as previously described (2) but never took the lipid to complete dryness. Instead, methanol and water were removed by repeated addition of chloroform followed by evaporation to small volume. The moist lipid dissolved in chloroform-methanol (19:1) saturated with water was then applied to a Sephadex column for removal of nonlipid (3). Lipid in the first fraction from Sephadex was dissolved in a convenient volume of chloroform-methanol (9:1 or 2:1) and total lipid determined by weighing on a Cahn microbalance (4) the solids obtained from a small aliquot. The molar amounts of the phospholipids were determined by two-dimensional thin layer chromatography (TLC) and phosphorus analysis of spots (5).

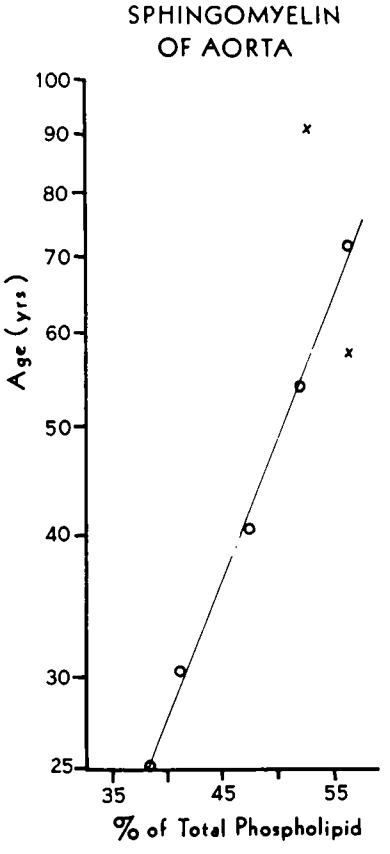

FIG. 1. A plot of sphingomyelin content of human male aorta at different ages. Open circles normal aortas and x's grossly sclerotic aortas (see text for details).

Postmortem enzymatic degradation results in release of free fatty acids from phospholipids and production of various lysophospholipids. The effect of such changes was evaluated. Table I shows minimal changes for bovine aorta when it stood at 23 C for 24 hr postmortem. TLC showed no visible rise in the level of free fatty acid after 24 hr standing nor a fall of phospholipid values. Human aortas obtained 6-18 hr postmortem also had a low level of free fatty acid, and there was no correlation between the number of hours of standing postmortem prior to freezing or extraction and the phospholipid distribution or free fatty acid level. Postmortem enzymatic degradation thus appears to be of little significance in studies of human aorta lipids.

There are distinct regional variations in phospholipid composition of human aorta (Table I). This was demonstrated as follows. An aorta from the heart to the point of bifurcation in the abdomen was cut longitudinally into two equal pieces. One of these was further divided into the ascending portion, the descending thoracic portion and the abdominal segment. Each part was analyzed separately and compared to the analysis of the other half of the aorta. The phospholipid values of the different segments of the aorta (Table I) were different which means that representative samples of the whole aorta are required for precise determination of age related changes. Subsequent analyses were of homogenates of samples of aorta from the heart to the major bifurcation in the abdomen.

We compared the lipid composition of eight carefully selected normal aortas, essentially free of vascular disease, with two that were grossly sclerotic. One of the sclerotic aortas (from a 58 year old man) had about half its surface covered with plaques and the other

TABLE I

Effect of Standing Postmortem and Regional Variations on Phospholipid Composition of Aorta[a]

	Bovine		Human			
	Fresh	After 24 hr at 23 C	Whole	Ascending	Descending Thoracic	Abdominal
Phosphatidyl choline	33.0 ±0.49	33.0 ±0.64	30.2 ±0.94	28.7 ±0.56	28.8 ±0.60	30.4 ±1.3
Phosphatidyl ethanolamine	24.4 ±0.17	23.4 ±0.27	17.4 ±0.66	17.3 ±0.23	16.4 ±0.35	19.5 ±1.3
Phosphatidyl serine	10.1 ±0.08	10.6 ±0.15	7.5 ±0.18	8.4 ±0.16	7.3 ±0.10	9.3 ±0.35
Phosphatidyl inositol	5.1 ±0.33	4.7 ±0.33	2.9 ±0.07	3.1 ±0.16	2.5 ±0.10	4.7 ±0.20
Phosphatidic acid	0.8 ±0.12	0.7 ±0.15	0.6	0.5 ±0.26	0.4 ±0.08	0.8 ±0.25
Diphosphatidyl glycerol	1.8	0.4	0.7	1.1 ±0.35	0.8 ±0.20	1.5 ±0.15
Sphingomyelin	22.2 ±0.68	22.0 ±0.55	38.1 ±0.52	39.3 ±0.57	41.1 ±0.55	31.7 ±1.3
Lysophosphatidyl choline	0.4	0.9	1.2 ±0.11	0.8 ±0.45	0.6 ±0.10	1.2 ±0.65
Other[b]	2.2	4.3	1.4	0.8	2.1	0.9

[a] Values as percentage of the lipid phosphorus ± standard deviation.

[b] The difference between total phosphorus and that of the lipid classes identified.

(from a 90 year old man) was almost entirely covered with lesions. Although phospholipid composition of the aorta changed markedly with advancing age (Table II), the changes were not related to the presence of atherosclerotic lesions. Sphingomyelin increased steadily with increase in age whereas most other phospholipids decreased. When the changes with age are considered, the level of sphingomyelin in the grossly sclerotic vessels does not appear to be greatly elevated.

A plot of the logarithm of age in years (from 25 to 72 years) versus the percentage of total sphingomyelin phosphorus of normal male aortas gave a straight line (Fig. 1) expressed by the following regression equation: per cent

TABLE II

Phospholipids of Normal and Atherosclerotic Human Aortas[a]

	Normal males						Normal females		Atherosclerotic males	
	1 day	25 yrs	31 yrs	41 yrs	54 yrs	72 yrs	17 yrs	24 yrs	58 yrs	90 yrs
Phosphatidyl choline	32.9 ±1.3	30.2 ±0.94	27.8 ±0.78	26.1 ±0.32	20.8 ±1.10	21.8 ±0.67	34.0 ±0.01	25.7 ±0.65	24.0 ±0.44	23.8 ±0.68
Phosphatidyl ethanolamine	24.2 ±0.29	17.4 ±0.66	14.1 ±0.38	5.9 ±0.16	11.6 ±0.70	10.0 ±0.40	20.8 ±0.50	14.2 ±0.55	7.3 ±0.23	3.5 ±0.30
Phosphatidyl serine	9.8 ±0.50	7.5 ±0.18	7.3 ±0.22	2.7 ±0.20	4.6 ±0.01	4.3 ±0.57	9.1 ±0.15	7.4 ±0.25	3.0 ±0.35	2.9 ±0.25
Phosphatidyl inositol	5.1 ±0.01	2.9 ±0.07	2.8 ±0.30	2.1 ±0.07	1.7 ±0.01	1.3 ±0.20	4.7 ±0.15	3.5 ±0.20	1.7 ±0.07	2.2 ±0.09
Phosphatidic acid	T	0.6	0.2	0.6	T	T	0.6 ±0.30	0.5	T	T
Diphosphatidyl glycerol	1.1	0.7	0.7 ±0.02	0.5	0.3	T	1.4 ±0.15	1.5	0.4	T
Sphingomyelin	15.3 ±0.01	38.1 ±0.52	42.1 ±0.01	47.1 ±1.20	51.5 ±1.00	55.7 ±0.75	26.3 ±0.01	29.6 ±0.53	55.1 ±0.45	51.9 ±0.50
Lysophosphatidyl choline	0.7	1.2 ±0.11	1.8 ±0.18	6.9 ±0.50	1.6 ±0.20	1.6 ±0.70	0.6 ±0.13	2.8 ±0.25	4.8 ±0.07	4.5 ±0.10
Other[b]	10.9	1.4	3.2	8.1	7.9	5.3	2.5	14.8	3.7	11.2
Sphingomyelin Calculated[c]	....	38.5	42.1	46.8	51.4	56.2				

[a] Values as mean with standard deviation when four or more determinations were made.

T, trace, detectable but not measurable.

[b] The difference between total phosphorus and that of the lipids shown.

[c] From equation percent sphingomyelin = 38.44 ($\log_{10}$ age years)—15.20 (see text).

sphingomyelin (as percentage of the lipid phosphorus) = 38.44 (log age in years)-15.20. Similar plots for the other major phospholipid classes also gave straight lines but with opposite slopes. Equations for the other phospholipids were: per cent phosphatidyl choline = 54.91-17.88 (log age in years); per cent phosphatidyl ethanolamine = 38.73-15.71 (log age in years); per cent phosphatidyl serine = 19.31-8.262 (log age in years); and per cent phosphatidyl inositol = 8.315-3.804 (log age in years).

The values for females did not fall on the line for males. Female aortas at any particular age appear to contain less sphingomyelin. When the phospholipid values for the aorta of a newborn infant are compared to those of adults, it is apparent that there is a greater rate of increase of sphingomyelin content from birth to about 20 years than there is from 20 years onward.

GEORGE ROUSER
ROBERT D. SOLOMON[1]
Division of Neurosciences and
Department of Pathology
City of Hope Medical Center
Duarte, California 91010

[1]Present address: Doctors Hospital of San Leandro, San Leandro, California 94578.

ACKNOWLEDGMENT

This work was supported in part by U. S. Public Health Grants NB 01847 and NB 06237.

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[Received December 2, 1968]

Isolation of Highly Purified Human and Bovine Brain Endothelial Cells and Nuclei and Their Phospholipid Composition

Pure preparation of organelles and individual cell types from brain are required for determination of their composition and metabolic characteristics. In this report, we describe a procedure for preparing highly purified endothelial cells (capillaries) and nuclei from human and bovine brains and present phospholipid compositions of typical preparations of these and renal glomeruli which demonstrate in particular a lack of organ and species variability for capillary structures.

The isolation procedure (Fig. 1) employs modifications of differential and density gradient centrifugation commonly used for separations. Special features of the procedure are: large scale preparation; Sephadex G-25 used for separation of nuclei and endothelial cells; and foam concentration and glass bead column filtration used for further purification of endothelial cells.

Endothelial cell preparations from bovine and human brains appear to be free of other brain structures as judged by phase contrast microscopy (Fig. 2), although some preparations contain fine glass bead particles derived from the glass bead columns. Electron microscopy (Fig. 3) shows the typical structural characteristics of isolated endothelial cells. Phase contrast microscopy of nuclei isolated from bovine brain (Fig. 4) show predominately larger neuronal nuclei with some smaller glial nuclei. Contaminating structures other than Sephadex particles are barely detectable. Electron microscopy also indicates bovine brain nuclei to be practically free of adhering contaminants. When applied to human brain, the procedure gives preparations largely of the smaller glial cell nuclei which can be seen to have cytoplasmic material adhering to the nuclear membrane (Fig. 5). It seems probable that the difference between bovine and human preparations arises largely from the unavoidable and more extensive postmortem changes in human brain. Although other investigators (1) have prepared highly purified nuclei, to our knowledge no one has previously prepared pure endothelial cells.

We extracted the lipids, separated them from nonlipid substances, and determined phospholipids by previously described procedures (2,3). The phospholipid values (Table I) from three different bovine brain endothelial cell preparations show constant composition. A pure endothelial cell preparation from human brain (column 4, Table I) showed a composition similar to that of bovine brain thus demonstrating a lack of species variability. Glycolipid was not detected in the purest preparations of

sphingomyelin (as percentage of the lipid phosphorus) = 38.44 (log age in years)-15.20. Similar plots for the other major phospholipid classes also gave straight lines but with opposite slopes. Equations for the other phospholipids were: per cent phosphatidyl choline = 54.91-17.88 (log age in years); per cent phosphatidyl ethanolamine = 38.73-15.71 (log age in years); per cent phosphatidyl serine = 19.31-8.262 (log age in years); and per cent phosphatidyl inositol = 8.315-3.804 (log age in years).

The values for females did not fall on the line for males. Female aortas at any particular age appear to contain less sphingomyelin. When the phospholipid values for the aorta of a newborn infant are compared to those of adults, it is apparent that there is a greater rate of increase of sphingomyelin content from birth to about 20 years than there is from 20 years onward.

GEORGE ROUSER
ROBERT D. SOLOMON[1]
Division of Neurosciences and
Department of Pathology
City of Hope Medical Center
Duarte, California 91010

[1]Present address: Doctors Hospital of San Leandro, San Leandro, California 94578.

ACKNOWLEDGMENT

This work was supported in part by U. S. Public Health Grants NB 01847 and NB 06237.

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[Received December 2, 1968]

Isolation of Highly Purified Human and Bovine Brain Endothelial Cells and Nuclei and Their Phospholipid Composition

Pure preparation of organelles and individual cell types from brain are required for determination of their composition and metabolic characteristics. In this report, we describe a procedure for preparing highly purified endothelial cells (capillaries) and nuclei from human and bovine brains and present phospholipid compositions of typical preparations of these and renal glomeruli which demonstrate in particular a lack of organ and species variability for capillary structures.

The isolation procedure (Fig. 1) employs modifications of differential and density gradient centrifugation commonly used for separations. Special features of the procedure are: large scale preparation; Sephadex G-25 used for separation of nuclei and endothelial cells; and foam concentration and glass bead column filtration used for further purification of endothelial cells.

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Isolation of Nuclei and Endothelial Cells[1]

Fresh brain (1.2-1.4 kg) cut into ¼-½ in. coronal sections, placed in soln. 1 [2], drained, gray matter removed, chopped in 1 liter soln. 2 [3], drained, homogenized (loose-fitting 200 ml Potter-Elvehjem type) [5] in soln. 3 [4], diluted to 3 liters with soln. 2 [3] and passed through a 10 mesh sieve [6]. Centrifugations as follows:

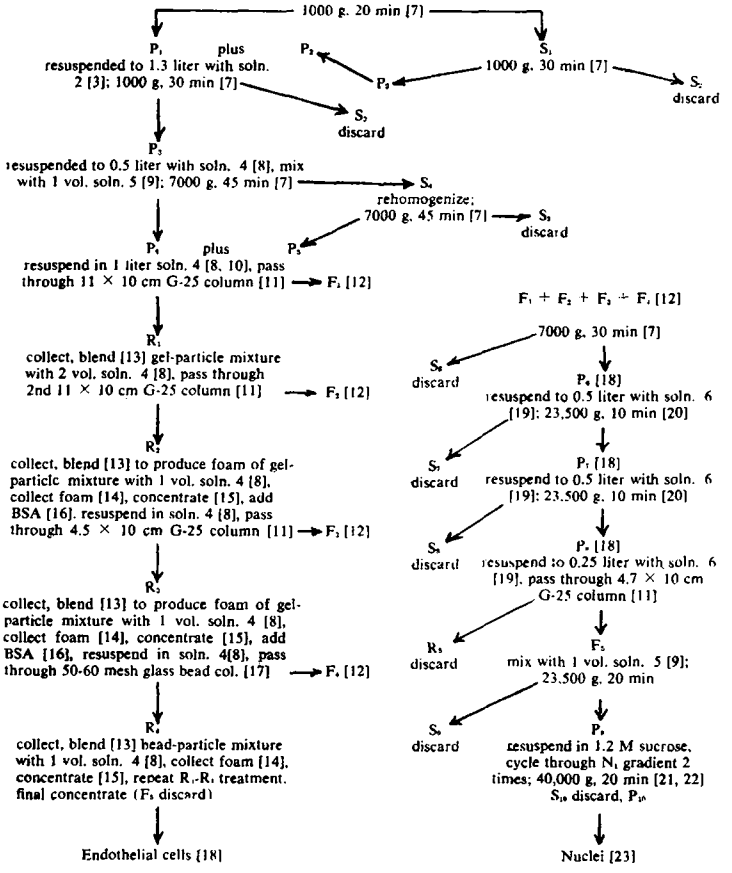

Fig. 1

[1] All centrifugations, manipulations and solutions at 4 C. Abbreviations: P, pellet; S, supernatant; R, visible residue in column bed; F, filtrate.
[2] Solution 1: 0.4 M sucrose containing 10,000 units of heparin.
[3] Solution 2: 0.4 M sucrose.
[4] Solution 3: 1 liter 0.4 M sucrose containing 20,000 units of heparin and 1 ml of 0.1 M adenosine triphosphate (ATP).
[5] 5-6 passes at 1200 rpm, 0.010 in. clearance.
[6] Final filtrate represents approximately 550 g of tissue.
[7] Sorvall RC-3, HG-4 rotor.
[8] Solution 4: 0.4 M sucrose containing 0.1 mM $CaCl_2$, 1 mM ATP, 0.5 mM 2-N-morpholino ethanesulfonic acid buffer (MES), pH 6.1.
[9] Solution 5: 2.0 M sucrose.
[10] Plus 1000 units heparin.
[11] Sephadex G-25, coarse, swell gel in solution 4 for 30-60 min; apply sample, pass 1 bed volume soln. 4 through, stir residue layer, repeat with two additional bed volumes.
[12] F_1, F_2, F_3, F_4 combined for recovery of nuceli.
[13] 1-2 sec in 500 ml Waring blendor.
[14] Repeat foam production (3-4 times) until foam free of endothelial cells.
[15] Centrifuge in 30 ml Corex centrifuge tubes, in HG-4 rotor, 1000 g, 10 min.
[16] 10% bovine serum albumin in 0.9% saline.
[17] Glass beads, 50-60 mesh (#090 Minnesota Mining & Manuf. Co.) packed in solution 4.
[18] Yield: 2-5 g.
[19] Solution 6: 0.83% NH_4Cl solution.
[20] Lourdes Model A, VRA rotor.
[21] N_1 gradient: linear, 1.2 M to 2.25 M sucrose.
[22] Spinco Model L-2, $SW_{25.2}$ rotor.
[23] Yield 1-2 g.

TABLE I

Phospholipid Composition of Bovine and Human Brain Endothelial Cells

Lipid Class[a]	Bovine Endothelial Cells[b] 1	2	3	Human Endothelial Cells 4	Human Glomerulic 5	Human Aorta[d] 6
Phosphatidyl choline	32.4 ±0.2	32.2 ±0.4	31.8 ±0.5	33.2 ±0.2	30.3 ±0.1	32.9 ±0.9
Phosphatidyl ethanolamine	23.5 ±0.2	24.6 ±0.2	23.6 ±0.4	25.2 ±0.4	24.2 ±0.1	24.2 ±0.3
Phosphatidyl serine	11.0 ±0.1	10.9 ±0.1	10.0 ±0.1	10.7 ±0.1	10.3 ±0.3	9.8 ±0.5
Phosphatidyl inositol	4.3 ±0.5	4.8 ±0.1	4.3 ±0.1	4.8 ±0.2	4.9 ±0.1	5.1 ±0.1
Phosphatidyl glycerol	0.9 ±0.1	0.8 ±0.2	0.7 ±0.1	ND	ND	ND
Diphosphatidyl glycerol	1.2 ±0.1	1.0 ±0.1	1.5 ±0.2	1.0 ±0.1	2.9 ±0.4	1.1 ±0.2
Phosphatidic acid	0.9 ±0.1	0.2 ±0.1	0.9 ±0.1	0.3 ±0.1	0.2 ±0.1	0.1 ±0.1
Lysobiphosphatidic acid	2.1 ±0.3	0.1 ±0.1	0.3 ±0.1	0.2 ±0.1	ND	T
Lysophosphatidyl choline	0.9 ±0.2	0.7 ±0.2	0.8 ±0.1	1.0 ±0.1	1.7 ±0.2	0.7 ±0.2
Sphingomyelin	20.4 ±0.3	21.2 ±0.2	21.0 ±0.2	17.0 ±0.1	21.1 ±0.3	15.3 ±0.3
Misc. minor	0.5 ±0.1	0.2 ±0.1	1.8 ±0.2	1.6 ±0.2	ND	10.7 ±0.3
Origin	0.3 ±0.1	0.1 ±0.1	0.3 ±0.1	0.2 ±0.1	0.3 ±0.1	0.2 ±0.1
Recovery (%)	98.4	96.8	97.0	97.2	97.7	100.1

[a] As percentage of the total lipid phosphorus ± standard deviation calculated from a minimum of 4 determinations. ND, not detected; T, trace.

[b] Three pure preparations from different brains.

[c] Isolated from kidney by filtration.

[d] Entire aorta from heart to major bifurcation in abdomen from 1 day old human male.

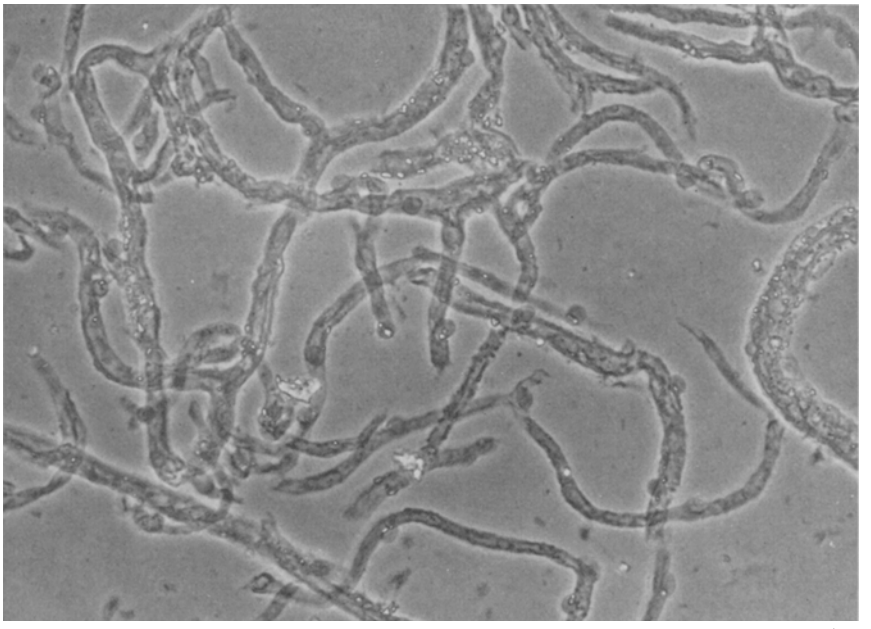

FIG. 2. Phase contrast micrograph of a human brain endothelial cell preparation (×200). The cells are covered with small lipofuscin granules, and capillary and small arteriolar structures can be seen.

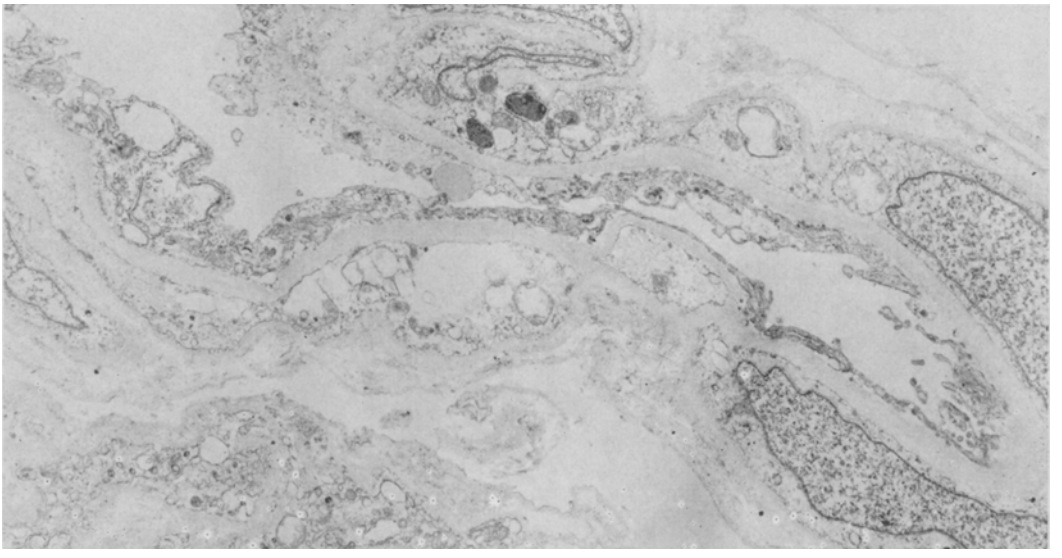

FIG. 3. Electron micrograph of human brain endothelial cells. In the center is a portion of a capillary. The capillary endothelial cell contains the lumen and is associated with pericytes; two are visible. The basement membranes of both pericytes and endothelial cells are clearly visible (×3600).

endothelial cells. The phospholipid composition of isolated human renal glomeruli (column 5, Table I) was very similar to that of brain endothelial cells. Thus, capillary structures from different organs appear to have the same lipid composition which is also like that of whole human aorta at birth (column 6, Table I).

The small variability in the phospholipid composition of bovine brain nuclei preparations (Table II) could be correlated with the purity of the preparations. Thus, preparation 1 (one of the most highly purified to date) had the lowest sphingomyelin, phosphatidyl serine, cerebroside and sulfatide levels, all of which indicate minimal contamination with myelin.

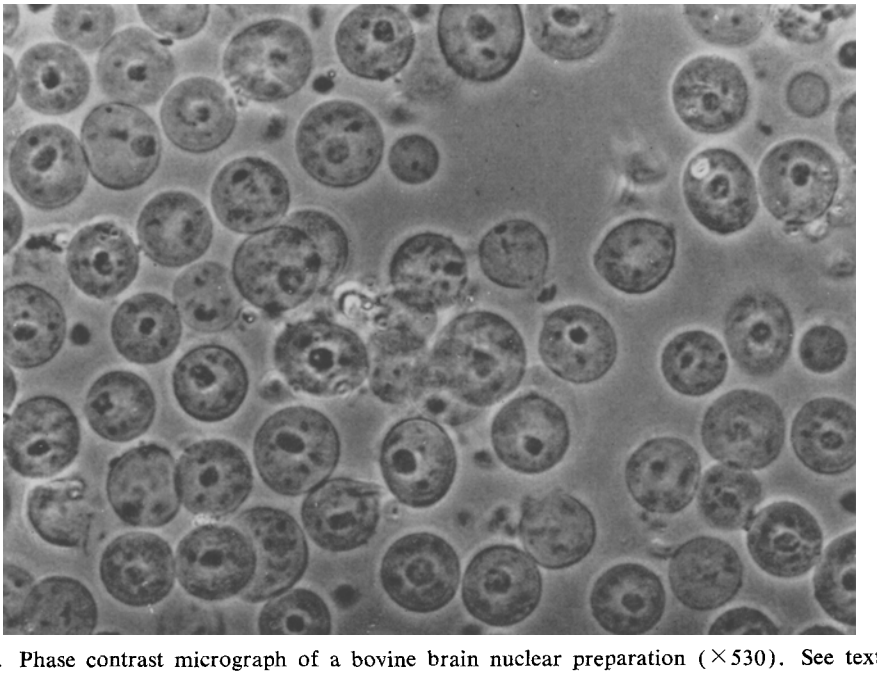

FIG. 4. Phase contrast micrograph of a bovine brain nuclear preparation (×530). See text.

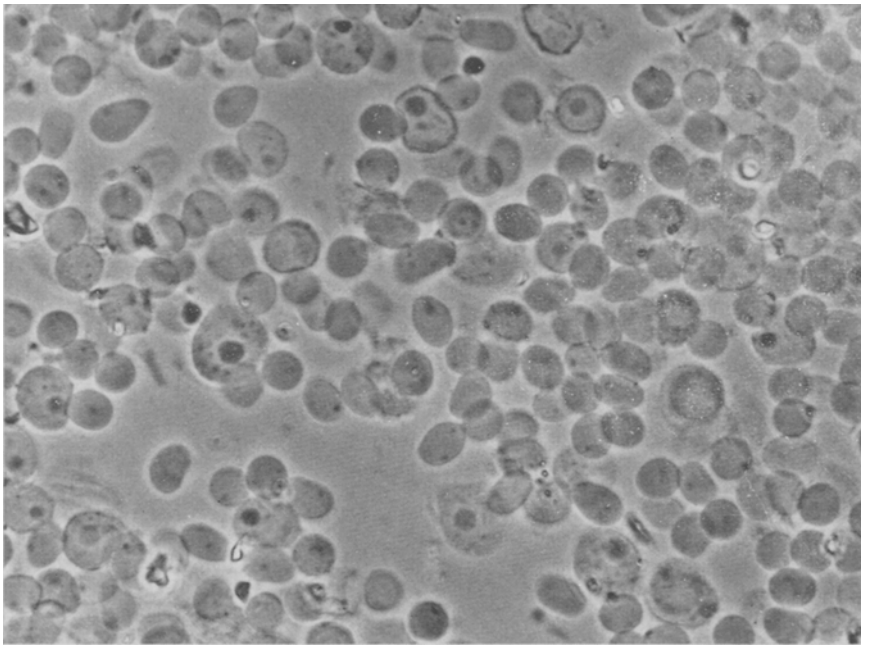

FIG. 5. Phase contrast micrograph of a human brain nuclear preparation (×530). See text.

TABLE II

Phospholipid Composition of Bovine and Human Brain Nuclei[a]

Lipid Class	Bovine[b] 1	2	3	Human[c]
Phosphatidyl choline	54.8 ±0.5	53.1 ±0.4	48.2 ±0.2	47.6 ±1.0
Phosphatidyl ethanolamine	24.2 ±0.7	20.8 ±0.4	23.8 ±0.1	17.8 ±0.4
Phosphatidyl serine	4.7 ±0.2	5.0 ±0.5	6.7 ±0.1	9.3 ±0.2
Phosphatidyl inositol	8.6 ±0.2	7.9 ±0.2	7.1 ±0.2	5.0 ±0.2
Phosphatidyl glycerol	T	T	0.4 ±0.2	0.6 ±0.2
Diphosphatidyl glycerol	0.5 ±0.1	ND	ND	0.3 ±0.1
Phosphatidic acid	0.4 ±0.1	0.6 ±0.1	1.3 ±0.2	1.2 ±0.2
Lysobisphosphatidic acid	0.2 ±0.1	1.1 ±0.2	0.7 ±0.2	0.2 ±0.1
Lysophosphatidyl choline	0.9 ±0.1	ND	1.0 ±0.2	2.5 ±0.1
Sphingomyelin	2.6 ±0.2	3.0 ±0.1	4.9 ±0.2	10.7 ±0.6
Misc. minor	2.4 ±0.2	7.5 ±0.2	2.8 ±0.2	2.0 ±0.2
Origin	0.1 ±0.1	0.8 ±0.1	0.2 ±0.1	0.6 ±0.2
Recovery	99.0	99.8	97.7	98.2

[a] Values as percentage of the total lipid phosphorus ± standard deviation calculated from a minimum of four determinations; ND, not detected; T, trace.

[b] Highly purified preparations.

[c] Contaminated with other structures.

Diphosphatidyl glycerol is a characteristic mitochondrial lipid (4,5) and thus its presence at a low level in preparation 1 indicates little contamination with mitochondria. No diphosphatidyl glycerol was detected in preparations 2 and 3, although preparation 3 did have somewhat higher levels of sphingomyelin, phosphatidyl serine and glycolipids indicating a greater contamination with myelin. The lipid composition of pure preparations of bovine brain nuclei was very similar to that found for nuclei of other organs (4). There thus appears to be little organ variability in lipid composition as noted also for mitochondria (4,5), although the lipid composition of mitochondria is characteristic and different from that of nuclei. The best human brain nuclei preparations are more contaminated with material of non-nuclear origin as judged by microscopic examination and the higher levels of sphingomyelin, phosphatidyl serine and glycolipid, presumably derived from myelin. In general, lipid analysis appears to be a valuable means for judging purity of fractions when used in conjunction with microscopic examination.

Phospholipid compositions of our purest preparations of nuclei differ significantly from the values reported for guinea-pig brain (6). These differences may be explained by the presence of endothelial cells, myelin and mitochondria in the nuclear preparations analyzed

by the previous investigators. These contaminants are suggested by the higher sphingomyelin values (high in myelin and endothelial cells), lower values for phosphatidyl choline, and the presence of diphosphatidyl glycerol (from mitochondria) which was not detected in extracts of most of our bovine brain nuclear preparations. The present lipid values for endothelial cells and glomeruli appear to be the first reported. The availability of a rapid procedure (2-3 hr) for isolation of highly purified endothelial cells provides a novel approach to the study of cerebral metabolism and allows direct study of the composition and permeability of these cells and factors influencing their metabolic activity.

A. N. SIAKOTOS
Department of Pathology
Indiana University Medical Center
Indianapolis, Indiana 46207
GEORGE ROUSER
Section of Lipid Research
Division of Neurosciences
City of Hope Medical Center
Duarte, California 91010

SIDNEY FLEISCHER
Department of Molecular Biology
Vanderbilt University
Nashville, Tennessee 37203

ACKNOWLEDGMENT

Supported by U.S. Public Health Grants NB 01847, NB 06237, GM 12831, and a grant-in-aid from the American Heart Association of which S. Fleischer is also an Established Investigator. Valuable aid in electron microscopy was provided by Akitsuga Saito and Hidehiro Ozawa.

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[Received December 2, 1968]

Phospholipid Composition of Human, Bovine and Frog Myelin Isolated on a Large Scale From Brain and Spinal Cord

Myelin has long been considered as a model for membrane structure studies. Furthermore, the amount of myelin is reduced in several pathological states in which it is essential to know whether the composition of myelin is normal or altered. Our studies of the structures (including lipid composition) of membranes from human and large animal brains necessitated development of an isolation procedure yielding highly purified myelin from larger quantities of tissue than usually employed. This report presents a large-scale isolation procedure for highly purified myelin from human, bovine and frog central nervous system. Also presented are the phospholipid class compositions of typical myelin preparations which show that mammalian and amphibian myelins differ in phospholipid composition.

The procedure (Fig. 1) has three special features. First, filtration through glass beads removes fibrillar structures encountered particularly in human brains showing degenerative changes. Second, improved separation of contaminants is achieved in one step by centrifugation in slightly acidic buffer and in another with the use of a Diodrast-sucrose gradient. Third, the volumes of suspensions are reduced rapidly by treatment with dextran gel rather than by centrifugation. With our procedure, both light and heavy myelin are isolated in gram quantities from human and bovine brain and spinal cord. The procedure was also applied successfully to frog brain and spinal cord.

Electron microscopy disclosed that heavy myelin (Fig. 2) has the lamellar structure commonly associated with myelin, whereas light myelin (Fig. 3) is composed of vesicles which are generally larger and less dense than microsomal vesicles from the endoplasmic reticulum. It is estimated from recovery values that about 80% of the myelin from spinal cord and 20% of the myelin from cerebral white matter vesiculated to form light myelin, whereas cerebral cortical grey matter homogenates gave almost entirely heavy myelin.

Phospholipid values for brains of adult animals (Table I) were obtained after extraction (1) and removal of nonlipid contaminants (1,2) by phosphorus analysis of spots separated by two-dimensional thin layer chromatography (3). Significant and variable enzymatic breakdown is indicated by values of 0.3-7.9% for lysophosphatidyl ethanolamine and the variable amount of the total phosphorus that was not

by the previous investigators. These contaminants are suggested by the higher sphingomyelin values (high in myelin and endothelial cells), lower values for phosphatidyl choline, and the presence of diphosphatidyl glycerol (from mitochondria) which was not detected in extracts of most of our bovine brain nuclear preparations. The present lipid values for endothelial cells and glomeruli appear to be the first reported. The availability of a rapid procedure (2-3 hr) for isolation of highly purified endothelial cells provides a novel approach to the study of cerebral metabolism and allows direct study of the composition and permeability of these cells and factors influencing their metabolic activity.

A. N. SIAKOTOS
Department of Pathology
Indiana University Medical Center
Indianapolis, Indiana 46207
GEORGE ROUSER
Section of Lipid Research
Division of Neurosciences
City of Hope Medical Center
Duarte, California 91010

SIDNEY FLEISCHER
Department of Molecular Biology
Vanderbilt University
Nashville, Tennessee 37203

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Isolation of Heavy and Light Myelin [1].

Cut fresh whole brain (1.2-1.4 kg) into ¼-½ in. coronal sections; place in solution 1 [2]; transport to the laboratory; drain; separate grey and white matter; chop in solution 2 [3]; drain; adjust solution volume by dilution of each gram of homogenate to 2 ml with solutions 3 [4]; homogenize in a loose-fitting 200 ml Potter-Elvehjem homogenizer [5]; dilute to 10 ml/g with solution 2 [3]; pass through a 10 mesh aluminum sieve; add ¼ volume solution 4 [6] and mix. Centrifuge as follows:

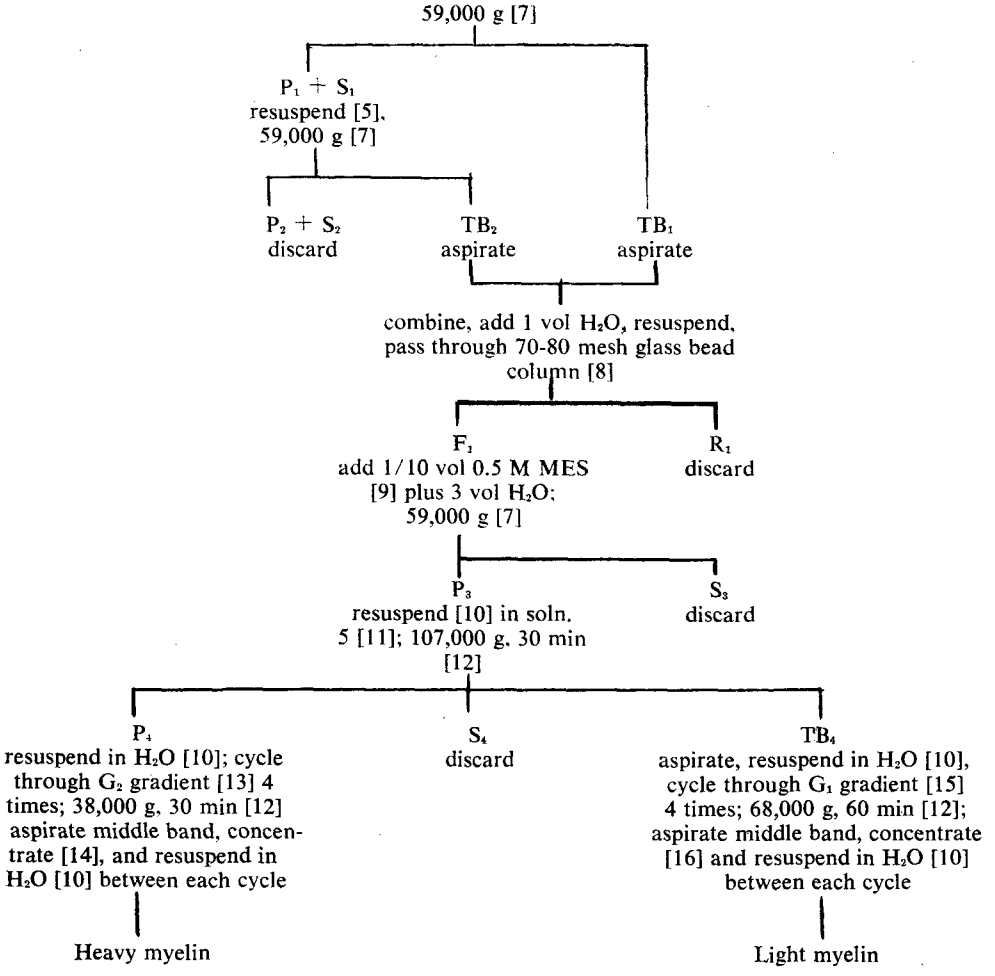

Fig. 1

[1] Centrifugations, manipulations and solutions at 4 C. Abbreviations: P, pellet; S, supernatant; TB, top band; F, filtrate; R, residue.
[2] Solution 1: 1 liter 0.4 M sucrose containing 10,000 units heparin.
[3] Solution 2: 0.4 M sucrose.
[4] Solution 3: 1 liter 0.4 M sucrose containing 20,000 units heparin.
[5] 5-6 passes at 1200 rpm.
[6] Solution 4: 2.0 M sucrose.
[7] Spinco L-2 #21 rotor, 30 min from start to max. speed.
[8] Hydrochloric acid washed glass beads, 70-80 mesh (#100, Minnesota Mining and Manuf. Co.) packed in sol. 2 [3].
[9] MES (2-N-morpholino ethanesulfonic acid) buffer, pH 6.1.
[10] Homogenizer with 0.007 in. clearance.
[11] Solution 5: 5% Diodrast (Iodopyracet, 35%, w/v; Winthrop Labs., N. Y.) in 0.4 M sucrose.
[12] Spinco L-2, $SW_{25.2}$ rotor.
[13] G_2 gradient: linear, 0.4 M sucrose to 10% Diodrast in 0.4 M sucrose.
[14] Add 1 vol. solution 2 [3] and centrifuge 23,500 g, 20 min, Lourdes Model A, VRA rotor (1.5 liter vol.).
[15] G_1 gradient: linear, distilled water to 0.4 M sucrose.
[16] Add 4 vol. distilled water, mix, add 260 g dry Sephadex G-25, coarse, per liter, allow to stand 15-30 min, collect free fluid by centrifugation (500 g).

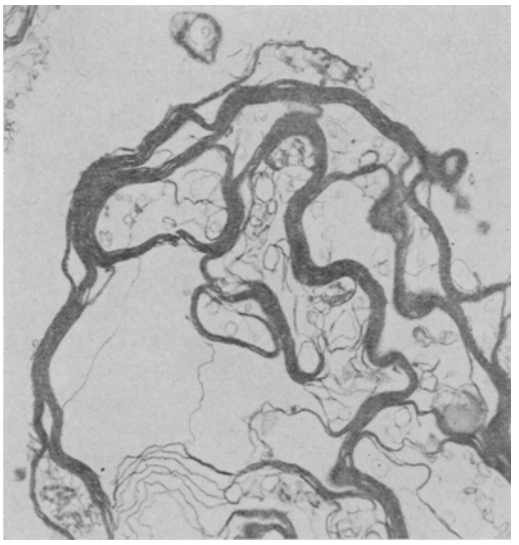

FIG. 2. Electron micrograph (×12,000) of heavy myelin (see text for details).

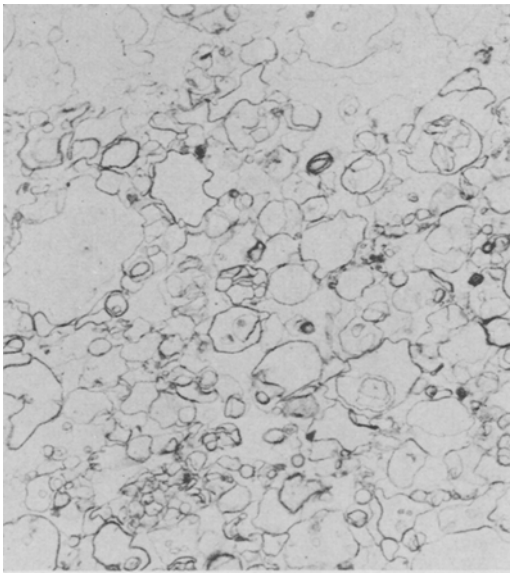

FIG. 3. Electron micrograph (×15,000) of light myelin (see text for details).

positively identified. The effect of these variables is minimized (Table II) when the sum of the values for phosphatidyl ethanolamine and lysophosphatidyl ethanolamine is used as the correct value for phosphatidyl ethanolamine and the values for the identified lipid classes are taken as 100% (unidentified phosphorus-containing substances eliminated). The similarity of values for human brain and spinal cord thus becomes apparent. Furthermore, values for

TABLE I

Phospholipid Composition of Myelin

(Values as percentage of total lipid phosphorus)[a]

	Human					Bovine				Frog
Lipid Class	Brain[b] grey matter (heavy)	Brain[b] white matter (heavy)	Brain[b] mixed grey & white (heavy)	Brain[b] mixed grey & white (light)	Spinal cord (light)	Brain white matter (heavy)	Brain mixed grey & white (light)	Brain mixed grey & white (heavy)	Spinal cord (light)	Mixed brain & spinal cord (heavy)
Phosphatidyl choline	21.0 ±0.4	20.4 ±0.8	20.5 ±0.5	21.8 ±0.3	18.6 ±0.6	18.8 ±0.3	19.1 ±0.4	18.4 ±0.2	19.7 ±0.2	30.6 ±0.4
Phosphatidyl ethanolamine	30.6 ±0.3	27.7 ±1.0	33.8 ±0.5	35.4 ±0.6	36.8 ±0.5	37.2 ±0.3	40.8 ±0.9	36.1 ±0.5	38.4 ±0.5	37.0 ±0.7
Phosphatidyl serine	20.5 ±0.2	17.8 ±2.4	18.6 ±0.6	18.8 ±0.6	15.9 ±0.5	19.1 ±0.3	15.5 ±0.2	18.0 ±0.4	15.3 ±0.3	7.1 ±0.4
Phosphatidyl inositol	2.0 ±0.4	1.9 ±0.1	1.7 ±0.3	1.8 ±0.5	1.6 ±0.3	1.0 ±0.3	1.1 ±0.2	1.8 ±0.2	1.2 ±0.1	1.6 ±0.5
Phosphatidic acid	0.5 ±0.05	0.6 ±0.2	1.3 ±0.2	1.1 ±0.2	1.4 ±0.2	1.5 ±0.1	1.1 ±0.2	1.7 ±0.1	1.3 ±0.1	3.0 ±0.1
Sphingomyelin	11.7 ±0.5	12.7 ±0.6	15.0 ±0.4	16.3 ±0.5	20.7 ±0.5	14.6 ±0.4	18.7 ±0.1	15.0 ±0.2	17.6 ±0.8	4.8 ±0.2
Lysophosphatidyl ethanolamine	5.5 ±0.2	7.9 ±0.7	2.2 ±0.3	2.0 ±0.2	0.3 ±0.2	0.7 ±0.1	0.6 ±0.2	0.2 ±0.1	ND	2.4 ±0.1
Lysobisphosphatidic acid	0.3 ±0.2	0.8 ±0.3	1.0 ±0.3	1.0 ±0.2	0.3 ±0.2	ND	0.2 ±0.1	0.4 ±0.1	0.9 ±0.2	ND
Sum[c]	92.1	89.8	94.1	98.2	95.6	92.9	97.1	91.6	94.4	86.5
% P recovered[d]	96.9	100.8	97.2	99.6	100.6	99.9	100.8	100.0	99.7	97.1
No. determinations	4	8	4	4	4	8	4	4	4	4

[a] ± Standard deviation.
[b] From the same brain.
[c] Sum of all identified lipids.
[d] Per cent of total phosphorus applied.
ND, not detected.

TABLE II
Phospholipids of Myelin
(Values converted for uniform basis of comparison)[a]

Lipid class	Human					Bovine				Frog
	Brain grey matter (heavy)	Brain white matter (heavy)	Brain mixed grey & white (heavy)	Brain mixed grey & white (light)	Spinal cord (light)	Brain white matter (heavy)	Brain mixed grey & white (light)	Brain mixed grey & white (heavy)	Spinal cord (light)	Mixed brain & spinal cord (heavy)
Phosphatidyl choline	23.0	22.8	21.9	22.2	19.5	20.3	20.9	22.2	20.8	36.5[b]
Phosphatidyl ethanolamine	39.1	39.6	38.3	38.0	38.6	40.8	42.0	44.5	40.6	44.7
Phosphatidyl serine	22.2	19.7	19.6	19.3	16.7	20.5	21.1	22.4	16.2	8.1
Phosphatidyl inositol	2.2	2.1	1.8	1.9	1.7	1.1	1.1	1.2	1.3	1.8
Phosphatidic acid	0.5	0.8	1.4	1.1	1.5	1.6	1.6	1.7	1.4	3.4
Sphingomyelin	12.7	14.1	15.9	16.5	21.7	15.7	16.1	17.1	18.7	5.5
Lysobisphosphatidic acid	0.3	0.9	1.1	1.0	0.3	ND	ND	ND	1.0	ND

[a] Uncharacterized substances eliminated so that the lipid classes shown total 100% and the sum of the values for phosphatidyl ethanolamine plus lysophosphatidyl ethanolamine is entered as phosphatidyl ethanolamine.

[b] Sum of phosphatidyl choline and lysophosphatidyl choline values.

bovine brain and spinal cord are similar to each other and to the values for human brain and spinal cord myelin. In contrast, frog myelin contains more phosphatidyl choline and phosphatidyl ethanolamine and less sphingomyelin and phosphatidyl serine than human or bovine myelin.

Although the phospholipid values obtained for our myelin preparations are distinctly different from those obtained by earlier investigators (4-6) who used different procedures for isolation and analysis, the general conclusions from our studies are in good agreement with those of some previous reports. Thus, the close similarity of light and heavy bovine brain myelin was reported by Norton and Autilio (4) and the close similarity of myelin lipid composition of several mammalian species and was emphasized by Evans and Finean (5) who presented data for several species and compared their values to those previously reported. Our data for frog myelin demonstrate in addition that myelin from a lower vertebrate has a different phospholipid composition from mammalian myelin.

A. N. SIAKOTOS
Department of Pathology
Indiana University Medical Center
Indianapolis, Indiana 46207

GEORGE ROUSER
Section of Lipid Research
Division of Neurosciences
City of Hope Medical Center
Duarte, California 91010

SIDNEY FLEISCHER
Department of Molecular Biology
Vanderbilt University
Nashville, Tennessee 37203

ACKNOWLEDGMENTS

Supported by U.S. Public Health Grants NB-01847, NB-06237, GM 12831, and a grant-in-aid from the American Heart Association of which S. Fleischer is also an Established Investigator. Aid in electron microscopy was provided by Akitsuga Saito and Hidehiro Ozawa.

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[Received December 23, 1968]

Variations Among Vertebrates of Lung Phospholipid Class Composition

Species variations in membrane lipid class composition can be studied by analysis of highly purified subcellular particulates and more rapidly by analysis of whole organs. This report presents analyses of lipid classes in human, bovine, rat, mouse, and frog lungs. The close similarity of the values obtained shows that, among vertebrates, there is little species variation of lung phospholipid composition.

Representative samples of whole human and bovine lung were obtained from homogenates. Several lungs from small animals were pooled and extracted. Lipids were extracted with chloroform-methanol (2:1 and 1:2 v/v) (1) and nonlipid contaminants removed by Sephadex column chromatography (1,2). Phospholipids were determined in Sephadex fraction 1 by phosphorus analysis of spots after separation by two-dimensional thin layer chromatography (3). Table I shows only small variations in composition when lung lipids of several individual rats were analyzed. Likewise, variation in lipid composition for the species examined was also small. Only three values (phosphatidyl ethanolamine in human lung; phosphatidyl choline and sphingomyelin in bovine lung) were distinctly outside the range observed for different samples of rat lung. Even these relatively

TABLE I

Phospholipid Composition of Vertebrate Lungs[a]

	Rat[b]					Mouse[c]	Frog[d]	Human[e]	Bovine
Sample No.	1	2	3	4	5	6	7	8	9
No. of Animals	(3)	(1)	(1)	(1)	(1)	(12)	(8)	(1)	(1)
% H_2O	77.72	80.51	78.94	75.10	75.37	80.46	85.00	89.99	78.32
% Lipid[f]	3.76	3.49	3.68	3.92	3.10	3.80	3.90	1.22	3.2
Lipid P[g]	1.65	1.64	1.40	1.30	1.80	1.70	1.51	1.14	1.51
Phosphatidyl choline	48.1 ±0.4	45.3 ±0.3	42.7 ±0.4	43.8 ±0.3	44.7 ±0.3	43.7 ±1.2	42.6 ±1.0	47.5 ±0.5	39.5 ±0.5
Phosphatidyl ethanolamine	21.4 ±0.2	23.7 ±0.3	21.7 ±0.5	22.3 ±0.3	22.1 ±0.0	20.1 ±0.2	21.2 ±0.5	17.5 ±0.4	21.2 ±0.6
Phosphatidyl serine	9.0 ±0.1	8.6 ±0.1	9.1 ±0.2	9.3 ±0.3	9.0 ±0.4	8.1 ±0.3	7.8 ±0.0	7.0 ±0.3	9.4 ±0.2
Phosphatidyl Inositol	4.0 ±0.1	4.2 ±0.1	3.8 ±0.2	3.9 ±0.1	3.7 ±0.1	4.1 ±0.2	3.4 ±0.2	3.2 ±0.1	3.3 ±0.1
Phosphatidic acid	0.1 ±0.05	0.2 ±0.1	0.2 ±0.1	0.4 ±0.2	0.5 ±0.2	0.4 ±0.2	0.3 ±0.1	0.5 ±0.1	1.4 ±0.0
Diphosphatidyl glycerol	0.7 ±0.2	1.2 ±0.1	1.1 ±0.0	1.1 ±0.1	1.2 ±0.1	1.1 ±0.2	0.9 ±0.1	1.0 ±0.1	1.0 ±0.1
Phosphatidyl glycerol	2.6 ±0.3	2.4 ±0.2	2.1 ±0.0	1.6 ±0.3	2.1 ±0.1	2.4 ±0.2	1.8 ±0.2	2.5 ±0.2	2.0 ±0.1
Lysobisphosphatidic acid	0.4 ±0.1	0.3 ±0.1	0.2 ±0.1	T	0.4 ±0.1	0.3 ±0.1	0.3 ±0.1	1.5 ±0.1	0.5 ±0.2
Lysophosphatidyl choline	1.1 ±0.2	1.2 ±0.2	1.3 ±0.1	1.6 ±0.1	1.5 ±0.2	1.1 ±0.2	0.7 ±0.3	2.0 ±0.1	0.3 ±0.1
Lysophosphatidyl ethanolamine	0.5 ±0.2	ND	ND	T	ND	ND	0.2 ±0.1	0.6 ±0.1	0.2 ±0.1
Sphingomyelin	10.4 ±0.1	9.6 ±0.1	10.1 ±0.3	9.9 ±0.1	10.1 ±0.2	9.3 ±0.3	10.8 ±0.3	11.1 ±0.3	16.1 ±0.7
Sum	98.3	96.7	92.3	93.9	95.3	90.6	90.0	94.4	94.9
Unidentified	0.3	0.5	3.7	0.6	1.2	2.5	3.6	2.6	2.7
% P recovery	98.6	97.2	96.0	94.5	96.5	93.1	93.6	97.0	97.6
No. Determinations	4	4	4	4	4	8	12	8	8

[a] As percentage of the total lipid phosphorus (Sephadex fraction 1) ± standard deviation.

[b] Wistar strain.

[c] Swiss-Webster.

[d] *Rana pipiens* (similar results were obtained with the toad, *Bufo boreas*).

[e] Obtained 1 hr 45 min postmortem.

[f] Per cent fresh weight.

[g] Milligram phosphorus/100 mg lipid.

T, trace (detectable but too little to determine).
ND, not detected.

small differences may have been produced by postmortem enzymatic breakdown from the greater length of time required to obtain and work up the large human and bovine organs. Consistent with this interpretation was the finding that when part of human lung homogenate stood at 23 C for 12 hr the value for phosphatidyl ethanolamine was about 15% lower than that of another portion maintained in the frozen state.

CLAUDE F. BAXTER
GEORGE ROUSER[1]
GERALD SIMON[2]
Neurochemistry Laboratories
Veterans Administration Hospital
Sepulveda, California 91343

[1]Section of Lipid Research, Division of Neurosciences, City of Hope Medical Center, Duarte, California 91010.
[2]Department of Neurology, Presbyterian-St. Luke's Hospital, Chicago, Illinois 60612.

ACKNOWLEDGMENTS

This work was supported in part by U.S. Public Health Service Grants NB-03743, NB-06237, and NB-06113 and State of Illinois Department of Mental Health Grant 17-720.

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[Received December 2, 1968]

14: Sphingosine, A Novel Sphingosine Base From *Procambarus Clarkii*

Seventeen naturally occurring sphingosine bases have been well characterized at this writing. The term, "sphingosine bases," is used here to mean isomers of 18:sphingosine, or sphing-4-enine in the newer IUB-IUPAC nomenclature. Most of these, as well as a few additional, less well characterized bases, were summarized three years ago by Karlsson and Holm (1), who were responsible for discovering a number of them. The remainder, more recently reported, are four branched chain bases (2-4), as well as a long suspected, but only recently rigorously characterized sphingadienine (5,6). These seventeen bases have been found in many tissues of many species and include saturated, unsaturated, hydroxylated and branched chain isomers whose chain lengths vary from 16 to 20 carbons.

We wish to report the discovery, in the crayfish *Procambarus clarkii*, of 14:sphingosine (tetradeca-4-sphingenine), and to present and discuss the results of initial studies of sphingosine base composition of crayfish nonganglioside sphingolipids.

Ventral cord (usually including brain) was removed by careful dissection from 100 crayfish. Accumulated cords were washed by several suspensions in about 5 ml of ice cold, 0.9% aqueous NaCl solution. After blotting on filter paper, total weight of washed ventral cord was found to be 3.0 g.

Total polar lipids, less gangliosides, were prepared by Folch extraction and wash (7), followed by separation on a column of activated, acid washed silicic acid, 100-200 mesh (Unisil, Clarkson Chemical Co., Williamsport, Pa.), as previously described (8).

Thin layer chromatography (TLC) was used to monitor the column separation. TLC analyses were performed on 20 x 20 cm glass plates, using a 250 μ thickness of silicic acid (Silica Gel H acc. to Stahl, E. Merck A. G., Darmstadt, Germany). Separations were carried out using chloroform-methanol-methylamine (30% aq.) (65:25:8) (10). Standard samples of all compounds identified were either previously prepared in this laboratory or purchased from Applied Science, State College, Pa. Sprays used for detection and partial identification of spots were phosphomolybdate blue (11) and 50% aqueous sulfuric acid followed by charring.

Sphingosine bases were liberated from total polar lipid by methanolysis, isolated and subjected to periodate oxidation for conversion to aldehydes and subsequent analysis by gas liquid chromatography (GLC). All of these techniques have been previously described (8).

GLC analyses were performed at various temperatures between 105 and 140 C, using an F & M Model 400 Biomedical Gas Chromatograph equipped with a hydrogen flame ionization detector. A U-tube glass column, 183 cm x 4 mm i.d., packed with 4% diethylene glycol succinate (DEGS) on Gaschrom P (Applied

small differences may have been produced by postmortem enzymatic breakdown from the greater length of time required to obtain and work up the large human and bovine organs. Consistent with this interpretation was the finding that when part of human lung homogenate stood at 23 C for 12 hr the value for phosphatidyl ethanolamine was about 15% lower than that of another portion maintained in the frozen state.

CLAUDE F. BAXTER
GEORGE ROUSER[1]
GERALD SIMON[2]
Neurochemistry Laboratories
Veterans Administration Hospital
Sepulveda, California 91343

[1]Section of Lipid Research, Division of Neurosciences, City of Hope Medical Center, Duarte, California 91010.
[2]Department of Neurology, Presbyterian-St. Luke's Hospital, Chicago, Illinois 60612.

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We wish to report the discovery, in the crayfish *Procambarus clarkii*, of 14:sphingosine (tetradeca-4-sphingenine), and to present and discuss the results of initial studies of sphingosine base composition of crayfish nonganglioside sphingolipids.

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Science, State College, Pa.), was employed.

Formation of bisulfite addition products, catalytic hydrogenation and $NaBH_4$ reduction were all performed as described in an earlier report (8).

TLC of the ganglioside free, total polar lipids showed the main constituents to be phospholipids, a minor component of which was sphingomyelin. Cerebroside and sulfatide were found present in very low, almost trace, quantities. Phosphatidyl serine was another minor component. The major polar lipid components were phosphatidyl choline and phosphatidyl ethanolamine. These observations are compatible with those of Reinisova and Michalec (12), who noted a steady relative decrease in central nervous system glycolipids in the direction of lower species.

GLC analysis of sphingosine bases revealed three major aldehyde peaks, two of which were identified as 14:sphingosine and 16:sphingosine, in a ratio of 59:41. The criteria used for identifying these were: (a) retention time identity with authentic standards at several widely differing temperatures, (b) retention time predictability from semilogarithmic carbon number plots, again at several temperatures, (c) formation of bisulfite addition products, (d) catalytic hydrogenation and (e) reduction by sodium borohydride. In cases (c), (d) and (e) disappearance of the original peak and reappearance of an appropriate peak upon subsequent GLC provide evidence for the identity of the aldehyde in question. 16:Sphingosine was positively identified by criteria (a) through (d), 14:sphingosine by criteria (b) through (d). The final aldehyde mixture was too impure, and in too small a quantity, for us to report other than corroborative and tentative, rather than definitive evidence, via criterion (e). The third peak meets the criteria for a small, unsaturated aldehyde, but cannot at present be identified. It is not likely to be an artifact and may represent yet another new sphingosine base; none of the presently known bases would run at the very low retention time exhibited by this aldehyde derivative. Trace quantities of 18:sphingosine and 18:dihydrosphingosine were also noted.

It is interesting to compare our data with that obtained by Reinisova and Michalec (12), who isolated sphingosine containing glycolipids from crayfish and investigated their sphingosine base composition using TLC of dinitrophenyl (DNP) derivatives. They found no 18:dihydrosphingosine present and reported finding three sphingosine bases, two of which were unidentifiable by their system, one of which was 18:sphingosine and was the major base present. The latter finding is in sharp disagreement with our data. Of the many possible explanations for the difference in data the best probably lies in the fact that these workers analyzed sphingosine containing glycolipids, which were a very minor part of the total sphingolipid contained in our polar lipid fraction. It is possible that crayfish sphingomyelin has a very different sphingosine base composition from that of cerebroside and sulfatide. We have noted significant differences between these two classes in recent work with other species (unpublished observations). In addition, it should be noted that TLC identification of DNP sphingosine base derivatives is not as reliable a method as the GLC technique, unless accompanied by many more controls than those available in the work cited above.

EZIO A. MOSCATELLI
KATHLEEN M. GILLILAND
Biochemistry Department
The University of Texas
Southwestern Medical School
Dallas, Texas 75235

ACKNOWLEDGMENTS

Ebert Ashby and Larry Jordan provided crayfish ventral nerve. This investigation was supported in part by Robert A. Welch Foundation Grant I-132 and USPHS Grant NB-06770-02.

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[Received December 18, 1968]

LETTER TO THE EDITOR

The Resolution of Complex Triglyceride Mixtures[1]

Sir: A number of excellent techniques for separating and analyzing triglycerides have been discovered in recent years, and it is possible to resolve most triglyceride mixtures into classes having the same carbon and double bond numbers. (The term class is used for glycerides having the same carbon and double bond numbers. The term group is used for glycerides having the same carbon or double bond numbers.) However, a complete analysis has been possible for only the simplest mixtures of triglycerides (McCarthy and Kuksis, JAOCS *41*, 527-530, 1964; Coleman, Ibid. *42*, 1040, 1965; Brockerhoff, Lipids *1*, 162, 1966; Lands and Slakey, Ibid. 295, 1966). For example Coleman (JAOCS *42*, 1040, 1965) has pointed out that, in the nut oils which include coconut and palm kernel oils, "The wide range in molecular weight of the fatty acids present, and their largely saturated character, offer very limited opportunities for separating their mixed triglycerides." He further points out that straightforward application of present techniques would leave over 100 possible triglycerides unresolved in these oils and that fish oils with their wide range of polyunsaturated acids "will clearly require radically different methods from those now in prospect, if they are to be analyzed exhaustively." This letter will point out that a complete triglyceride analysis can be obtained for these fats and most natural fats by: separating the triglyceride mixture into classes; forming diglycerides from these classes by a method that is random with respect to the acyl groups; separating the 1,3- from the 1,2- plus 2,3-diglycerides; separating either the 1,3- or 1,2- plus 2,3-diglycerides into classes; and stereospecific analysis of each of the diglyceride classes.

The application of this analytical scheme to coconut oil is illustrated in Figure 1. First the coconut oil is separated according to number of double bonds. This yields seven groups, all of which could be resolved by this scheme, but for simplicity only the separation of the complex mixture of trisaturated glycerides is given in detail. The saturated triglycerides are separated according to carbon number into 16 classes. Again for simplicity only carbon number 38, one of the most complex of the mixtures, is analyzed in detail. Diglycerides are generated by some random procedure and separated into the 1,3-diglycerides, the 1,2- plus 2,3-diglycerides, monoglycerides and unreacted triglycerides. Then either the 1,3- or 1,2- plus 2,3-diglycerides are refractionated according to carbon number. Consider the 1,3-diglycerides. All the diglycerides that suffered the loss of 18:0 will now have a carbon number 20, all that lost 16:0 will have a carbon number 22, etc., so there will be a different diglyceride class for each of the fatty acids lost from the 2 position. Examination of the 1,3-diglyceride classes shows that they are completely resolvable by stereospecific analysis, for each different acyl group occurs only once in the 1 and once in the 3 position. This will be true no matter how many acyl groups are involved, for each class has a fixed carbon number and a given acyl group can occur in combination with only one other to give the correct sum. Moreover, if an acyl group occurs twice (as 8:0 and 12:0 in class 20, for example) the two 1,3-diglycerides are optical isomers and arose from triglyceride optical isomers. Thus, if the distinction of optical isomers is not necessary, a simple analysis of the 1,3-diglyceride classes for their acyl groups will resolve the mixture. If one knows the amount of 8:0 in class 20, for example, one knows it is combined with 12:0 and that the triglyceride from which it came contained 18:0 on the 2 position. The calculation of the amount of 8:0,18:0,12:0 plus 12:0,18:0,8:0 in the original triglyceride mixture is straightforward. (The acyl groups are listed here as they occur on the 1, 2, and 3 positions of glycerol.) Likewise if one knows the amount of 8:0 on the 1 position from a stereospecific analysis of 1,3-diglyceride class 20, one can calculate the amount of 8:0,18:0, 12:0 in the original mixture.

If one works with the 1,2- plus 2,3-diglycerides, the same results can be achieved. There will be twice as many 1,2- plus 2,3-diglycerides as there were 1,3-diglycerides. If we consider again the optical isomers 8:0,18:0,12:0 and 12:0,18:0,8:0 and refer to Figure 1, we find that the diglycerides 8:0,18:0,- and -,18:0,8:0 derived from them occur in class 26 and the diglycerides -,18:0,12:0 and 12:0,18:0,- which are also derived from them, occur in class 30. Examination of the 1,2- plus 2,3-diglyceride classes, however, indicates that stereospecific analysis will still give a complete resolution of the mixture. Moreover, if an acyl group occurs

[1]Paper No. J-6060 of the Iowa Agricultural and Home Economics Experiment Station. Project 1517.

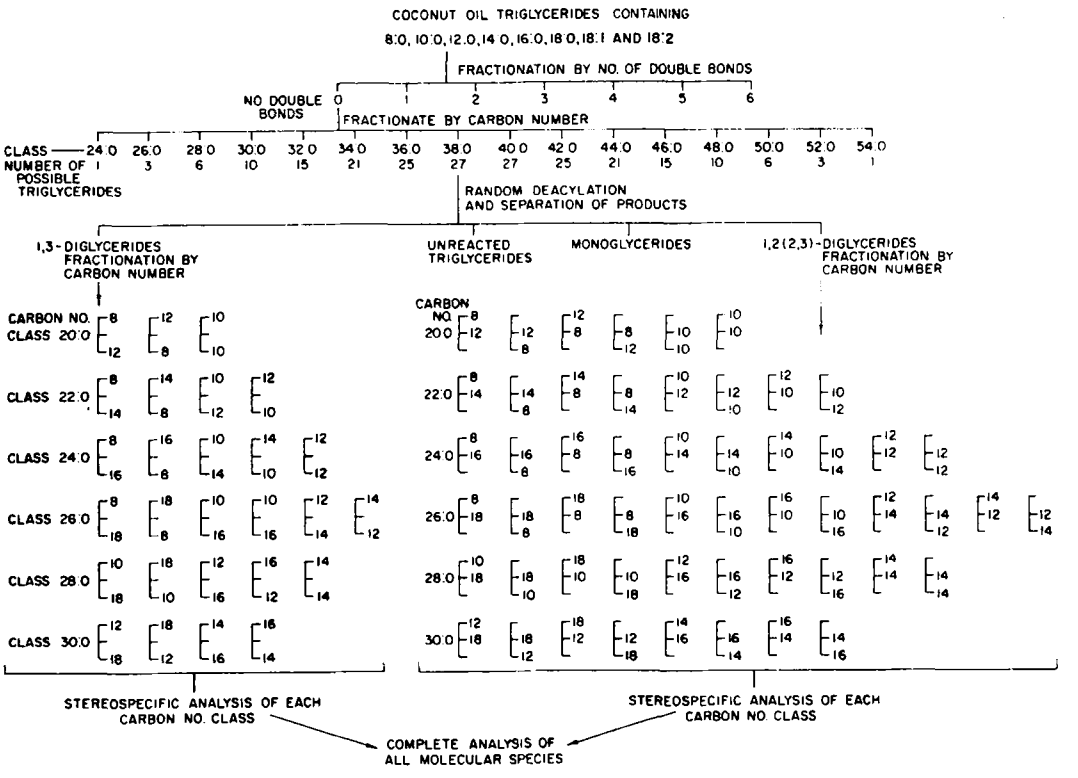

FIG. 1. Schematic resolution of the saturated triglycerides of coconut oil.

twice in a 1,2- plus 2,3-diglyceride class, on the 2 position, these diglycerides came from triglyceride optical isomers. This means that, if we ignore optical isomers, an analysis of the 2 position, such as obtained with pancreatic lipase (Mattson and Volpenhein, J. Lipid Res. *2*, 58-62, 1961), will give us a complete analysis. For example, in class 26 all the diglycerides with 18:0 on the 2 position came from 8:0,18:0,12:0 or 12:0,18:0,8:0. The same is true of the diglycerides containing 18:0 on the 2 position in class 30. The amounts of these diglycerides will allow the calculation of the amount of these two optical isomers in the original triglycerides.

Figure 2 illustrates how this same system would work for a hypothetical marine oil containing acyl groups of several chain lengths and degrees of unsaturation. For simplicity only the analysis of the triglyceride class of carbon number 54 and 6 double bonds is given in detail, and only the 1,3-diglycerides are written out. The only difference in this example and that of coconut oil trisaturated glycerides is that the diglycerides must be separated by degree of unsaturation as well as carbon number to give resolvable classes. From the diglyceride class carbon and double bond numbers, one may know the carbon and double bond numbers of the acyl group that has been removed. This allows calculation of the triglyceride composition of the original mixture as before.

Many variations of this separation scheme are possible. The separation into carbon number groups might be accomplished by gas liquid chromatography (Kuksis and Breckenridge, J. Lipid Res. *7*, 576-579, 1966; Litchfield, Harlow and Reiser, JAOCS *42*, 849-857, 1965; Kuksis and Ludwig, Lipids *1*, 202-208, 1966; Litchfield, Harlow and Reiser, Ibid. *2*, 363-370. 1967; Watts and Dils, J. Lipid Res. *9*, 40-51. 1968) or partition chromatography on thin layers or paper (Kaufmann and Wessels, Fette Seifen Anstrichmittel *66*, 81-86, 1964; Vereshchagin and Novitskaya, JAOCS *42*, 970-974, 1965). In partition chromatography methods, the apparent carbon number is changed according to the number of double bonds in the molecule, but one must always find the degree of unsaturation of the glyceride classes, so it is always possible to correct for the effect of the double bonds on the partition. The separation according to number of double bonds is best accomplished by silver ion chromatography

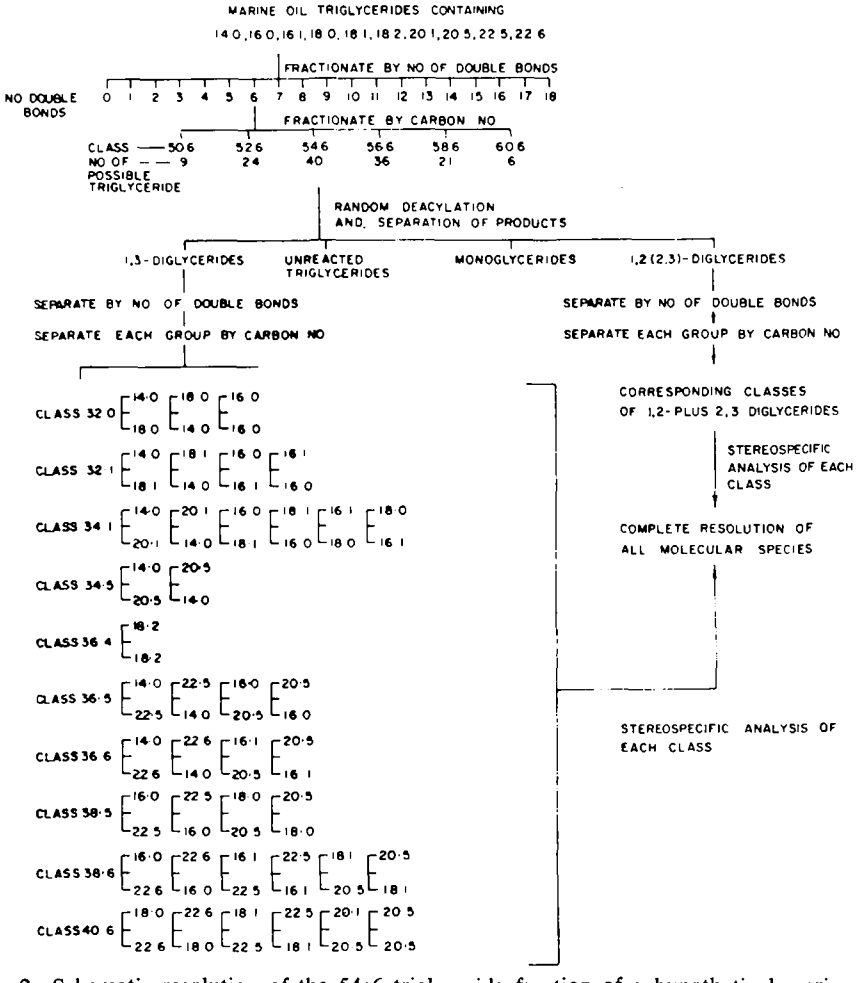

FIG. 2. Schematic resolution of the 54:6 triglyceride fraction of a hypothetical marine oil.

(Kaufmann and Wessels, Fette Seifen Anstrichmittel *66*, 81-86, 1964; Barrett, Dallas and Padley, JAOCS *40*, 580-584, 1963; DeVries, Ibid. *41*, 403-406, 1964; Litchfield, Farquhar and Reiser, Ibid. *41*, 589, 1964; Blank, Verdino and Privett, Ibid. *42*, 87-90, 1965). The order of separation by carbon number and double bond number may be reversed.

One could bring about the formation of diglycerides either by using Grignard reagent (Yurkowski and Brockerhoff, Biochem. Biophys. Acta *125*, 55-59, 1966) or pancreatic lipase (Mattson and Volpenhein, J. Lipid Res. *2*, 58-62, 1961; Anderson, Bottino and Reiser, Lipids *2*, 440-442, 1967). The latter, of course, would give only the 1,2- and 2,3-diglycerides, but this would not matter if there were no bias in the acyl group hydrolysis. There are satisfactory thin layer techniques for separating 1,2- plus 2,3-diglycerides, 1,3-diglycerides, monoglycerides and triglycerides from each other (Youngs and Subbaram, JAOCS *41*, 218-221, 1964; Thomas, Scharoun and Ralston, Ibid. *42*, 789-792, 1965). The migration of acyl groups from one position to another of diglycerides will limit the accuracy of the analysis. It may be desirable to block the free hydroxyl groups of the diglycerides with a suitable reagent as soon as separation of the 1,3- from the 1,2- plus 2-3-diglycerides has been achieved. This would prevent migration during the rest of the separation and might also facilitate the further fractionation of the diglycerides.

The stereospecific analysis of the 1,2 and 3 positions of glycerides may be accomplished by enzymatic methods (Lands and Slakey, Lipids *1*, 295, 1966; Lands, Pieringer, Slakey and Zschocke, Ibid. 444, 1966; Brockerhoff, J. Lipid Res. *6*, 10-15, 1965; Brockerhoff, Ibid. *8*, 167-

169, 1967).

Some glyceride classes will contain so few possible members that some of the separation and analysis steps may be omitted.

If acyl groups that differ in the position of double bonds are involved (such as oleoyl and petroselenoyl or α- and γ-linolenoyl) complete resolution is not always possible. Further information may be obtained by degradative analyses of the glyceride classes (Youngs and Subbaram, JAOCS, *41*, 218-221, 1964; Privett and Blank, J. Lipid Res. *2*, 37-44, 1961), but this would give complete resolution only if each unsaturated acyl group gave a unique degradation product that remained attached to the glycerol.

This method will lead to a great many fractions and the cleanness of the separations and the accuracy of the final analysis will have a great effect on the ability of the method to detect minor components. This method will be laborious and automation of the techniques and calculations will be required for its practical application on a routine basis.

In using this separation method it is helpful to know how many glyceride groups and classes may be expected and how many members are possible in each.

If one writes an equation in powers of X such that each chain length is represented by a power of X and cubes this expression, the coefficients of the powers of X corresponding to the carbon numbers of the triglycerides in the expanded expression will represent the number of possible triglycerides in this class. For example, if the fatty acids permitted have chain lengths of 14,16,18 and 20 the expression would be

$$(X^{14} + X^{16} + X^{18} + X^{20})^3$$

This is then multiplied out to give the powers of X representing triglyceride carbon numbers and the desired coefficients.

$$X^{42}+3X^{44}+6X^{46}+10X^{48}+12X^{50}+12X^{52}+10X^{54}+6X^{56}+3X^{58}+X^{60}$$

This tells us that there will be one possible triglyceride of carbon number 42, 3 of carbon number 44, 6 of carbon number 48, etc. If different degrees of unsaturation are included, we may write an expression

$$(X^{14:0} + X^{16:0} + X^{16:1} + X^{18:0} + X^{18:1} + X^{18:2} + X^{18:3} + X^{20:0})^3$$

In expanding this expression one must add the exponents in front of the colon and behind the colon separately. Then the coefficients of X corresponding to a given triglyceride carbon number (the number before the colon) and a given degree of unsaturation (the number after the colon) will be the number of triglycerides falling into this class. Each different carbon number and degree of unsaturation will yield a triglyceride class in the separation. During the cubing of the expression, one may keep track of the number of different exponents representing carbon number or double bond number that contribute to a triglyceride class. The number of different carbon or double bond exponents will be the number of diglyceride classes expected to be generated from that triglyceride class. The labor of multiplication may be minimized if one remembers that each set of three identical numbers (16+16+16) that add to a given carbon number will have a coefficient of 1, each set of three with two alike (14+14+20) will have a coefficient of 3, and each set of three with none alike (14+16+18) will have a coefficient of 6. If one does not wish to count optical isomers, change all the coefficients of 3 to 2 and all the coefficients of 6 to 3. For example, in the illustration given, the combinations that will occur in the expanded expression to give triglyceride class, 52:1 will be: 18:0+18:0+16:1, 18:0+18:1+16:0, 20:0+16:0+16:1, and 14:0+20:0+18:1. The coefficient of the first trio will be 3 and that of the others will be 6, so there are 21 possible triglycerides in this class. Or 11 if we ignore optical isomers. The exponents involved are: 18:0, 16:1, 18:1, 16:0, 20:0 and 14:0, a total of 6, so there will be 6 possible diglyceride classes from the triglyceride class 52:1.

ACKNOWLEDGMENTS

To Bernard Vinograde for suggestions about enumeration of glyceride classes and Carter Litchfield for helpful discussions and suggestions about this presentation.

E. G. HAMMOND
Department of Dairy and Food Industry
Iowa State University
Ames, Iowa 50010

[Received September 16, 1968]

Addendum

"Studies on Liver Phosphatidyl Cholines: I. Effects of Fatty Liver Induction on Phosphatidyl Cholines From Liver Mitochondria and Microsomes", James E. Miller and W. E. Cornatzer, The Gudy and Bertha Ireland Research Laboratory, University of North Dakota School of Medicine, Grand Forks, North Dakota 58201, Lipids 4, 19-27. In Table V, F-1 control value and F-1 choline deficient value for Fatty Acid 22:6 were misstated. Table should read as follows:

TABLE V

Fatty Acid Composition of Mitochondrial Phosphatidyl Choline Fractions From Livers of Female Rats Accumulating Fat

	Control[a]				Choline deficient[a]			
Fatty acid	F-1	F-2	F-3	F-4	F-1	F-2	F-3	F-4
14:0	0.1	0.3	0.2	0.1	0.2	0.4	0.2	0.4
15:0	0.1	0.1	t	0.1	0.1	0.2	0.2	0.2
16:0	17.4	12.9	13.3	16.8	20.5	14.9	16.9	29.2
16:1	t	1.8	0.8	1.0	0.5	1.4	1.2	2.6
17:0	0.6	0.5	0.4	0.5	0.2	0.5	0.9	1.9
18:0	28.3	23.6	33.1	31.5	17.6	19.9	22.8	16.3
18.1	1.4	10.8	4.1	19.7	4.2	9.3	5.8	26.2
18.2	3.5	7.5	9.8	24.7	1.8	5.4	5.7	19.5
18:3	.....	0.3	.....	0.3	0.3	1.5	0.7	0.6
20:3	t	t	0.2	1.6	0.9	4.8	1.2	1.0
20:4	3.8	24.0	38.1	3.4	2.9	12.4	43.4	1.9
20:5	1.0	10.3	.....	.....	1.4	10.9	.....	.....
22:5	.....	6.2	.....	.....	.....	10.2	.....	.....
22:6	43.7	1.8	.....	t	47.5	8.6	1.2	0.3
Others	0.1	1.8	.....	t	2.3	8.6	1.2	0.3

[a]Per cent of total fatty acid by weight.

(To readers of LIPIDS: The Lipids Advisory Board has recommended a policy, accepted by the AOCS Publications Committee, that the Editor of LIPIDS shall occasionally invite review articles on timely topics for publication in LIPIDS. The first such invitation was extended to Professor Dennis Chapman, who is an internationally recognized authority on the subject matter that he has covered in this review--Editor of LIPIDS.)

Physical Studies of Lipid-lipid and Lipid-protein Interactions

DENNIS CHAPMAN, Unilever Research Laboratory, The Frythe, Welwyn, Herts, Great Britain

ABSTRACT

This is a review of studies of lipid-lipid and lipid-protein interactions conducted in the author's laboratory. Studies of the thermotropic mesomorphism of phospholipids and effects due to the presence of water are described. The relevance of these thermal transitions to monolayer, bilayer and membrane systems is discussed. Studies on the interactions of phospholipids and cholesterol are reviewed. Finally, lipid-protein interactions under various conditions are discussed with special attention being given to serum lipoproteins and natural membranes.

INTRODUCTION

There is an increasing interest in understanding the nature of lipid-lipid and lipid-protein interactions. Lipid-lipid interactions are important to understanding the interactions of various lipids in emulsifiers, bile salt dispersions, and a variety of biological structures.

The interactions between phospholipids and proteins are fundamental in processes of biology and technology, and underlie a variety of important situations. The ways in which lipids and proteins interact in (a) cell membranes, (b) various enzyme reactions, e.g., with β-hydroxybutyrate dehydrogenase, (c) blood coagulation processes, (d) various lipase reactions, and (e) the structure of the serum lipoproteins, are among the many important areas under consideration and research at the present time(1).

The study of cell membrane structure raises many questions which illustrate the importance of understanding these interactions. Perhaps the most fundamental question is whether the bilayer model is a good one and whether it can be applied to all membrane types. This immediately raises fundamental questions about the nature of lipid-protein interactions in membrane structure(2).

If the bilayer or Davson-Danielli model is the correct structure for various membranes, a number of questions still require answers. For example, why does a particular cell membrane have characteristic distributions of alkyl chain lengths and unsaturation? How do the lipid chains interact with each other, and what is the degree of coiling or random character of these chains in a membrane? Why do some membranes contain a characteristic distribution of different lipid classes, and do the various polar groups of these lipids interact specifically with each other? Are the different lipids arranged in specific patterns or mosaics, or are they present in a random fashion? What is the nature of lipid-cholesterol interactions? Is the lipid-protein interaction electrostatic in character?

The lipids present in serum lipoproteins show more variations than those in cell membranes. In what ways do the lipids interact with each other? Is the nature of the lipid-protein interaction similar to that which occurs in cell membranes?

LIPID–LIPID INTERACTIONS

In our laboratory we have recently been examining some of the physico-chemical aspects of these questions. Because phospholipid molecules are principal lipid components of membranes and occur also in serum lipoproteins, our studies started with an examination of these molecules. We have investigated pure synthetic lipids, and are now extending our studies to interaction between lipids of different classes, chain lengths, and degrees of unsaturation.

Phospholipids

Thermotropic mesomorphism. Various phase changes occur in phospholipids at lower temperatures. When a pure phospholipid, e.g., dimyristoyl ethanolaminephosphatide, which contains two saturated acyl moieties is heated from room temperature to the capillary melting point, a number of thermotropic phase changes occur (i.e., phase changes caused by the effect of heat). This was shown by infra-red spectroscopy (IR) (3), by thermal analysis (4), and has

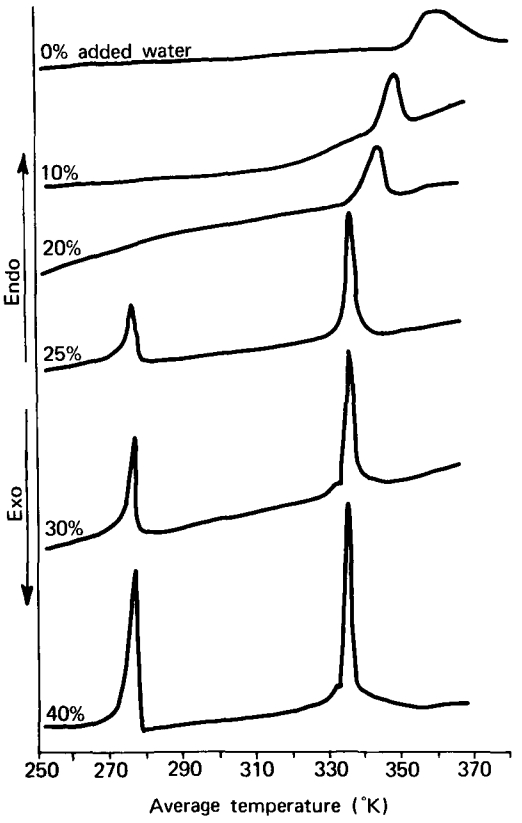

FIG. 1. DSC heating curves for 1,2-distearoylphosphatidylcholine in increasing amounts of water.

now been studied by a variety of other physical techniques (5,6).

The main conclusions from these studies are:

(1) Even in the saturated phospholipid, some molecular motion occurs in the solid state at room temperature. This is evident from the nuclear magnetic resonance (NMR) spectra, and from the IR spectra taken at room temperature and at the boiling point of liquid nitrogen.

(2) When the phospholipid is heated to higher temperature, it reaches a transition point, a marked endothermic change occurs, and the aliphatic chains "melt" and exhibit a very high degree of molecular motion. This is evident in the IR spectrum and also from the narrow NMR line width. The broad diffuse IR spectrum is consistent with the flexing and twisting of chains, and with a "break-up" of their all-planar *trans* configuration. That the phase transition involves primarily the aliphatic chains of the phospholipid is confirmed by X-ray analyses which show that the space taken up by the glycerol and polar group remains essentially unchanged when this phase transition occurs.

(3) Phospholipids that contain shorter carbon chains or unsaturated chains exhibit these marked endothermic phase transitions at lower temperatures (7). The temperatures at which these transitions occur fall in an order that parallels the melting points of the constituent fatty acids. The transition temperatures are high for the saturated long chain phospholipids, lower when there is a *trans* double bond present in one of the chains, and lower still when there is a *cis* double bond present.

(4) An important aspect of these phase transitions is that, above the endothermic transition temperatures, a given phospholipid will be in a highly mobile condition with its aliphatic chains flexing and twisting. This is a *fundamental property* of the phospholipid and we can expect that this chain mobility, unless somehow inhibited for special reasons, will normally be found in whatever situation the phospholipid occurs. Among such special reasons, we can envisage inhibition of chain motion by interaction with other molecules, such as water or protein. Due to less perfect packing arrangements at some temperatures, even greater mobility of the chains of the lipid and, indeed, diffusion of the whole lipid molecules, may be expected.

Effects of water.

(1) *Transition temperatures.* Small amounts of water can have unusual effects upon the mesomorphic behavior of phospholipids. Thus the phosphatidylcholines (lecithins) exhibit liquid crystalline forms between the first transition temperature and the capillary melting point (7). The intermediate liquid crystalline form is found to exhibit X-ray spacings consistent with a *cubic* phase organization. On the other hand, if all the water is removed from the phospholipid, it no longer exhibits this phase.

The marked endothermic transition temperature for a given phospholipid falls with increasing amounts of water (7) but it reaches a limiting value. We can understand this if we regard the effect of water as leading first to a "loosening" of the ionic structure of the phospholipid crystals. This, in turn, affects the entire crystal structure and a reduction, up to a certain limit, of the dispersion forces between the aliphatic chains. Quite high temperatures are still required to counteract the dispersion forces between the chains and to cause the chains to "melt." The limiting transition temperatures of individual phospholipids parallel the melting point behavior of their constituent fatty acids and are lower with increasing unsaturation. At the more common temperatures in biological systems, we should expect the phos-

pholipids which contain highly unsaturated chains *to be in a highly mobile and fluid condition.*

(2) *Bound water.* Some added water in phospholipid appears to be *bound* to the lipid; dipalmitoyl lecithin binds about 20% water (7). This water does not freeze at O C, and calorimetric studies made with lipid-water mixtures have shown that only after more than 20% has been added can one observe a peak at 0° C. Differential scanning calorimetry (DSC) curves of phospholipid-water mixtures in various ratios are shown in Fig. 1. This "bound" water may have considerable relevance to interactions of anesthetics, drugs, and ions with biological membranes. If the bound water varies either in its properties or its total amount, this may alter transport and diffusion properties across the membrane, depending on the type of interacting molecule or ion. The bound water associated with the constituent lipids and proteins will perhaps represent a minimum of water necessary for membranes to retain their organization. We are presently studying the bound water using D_2O and examination of the deuteron magnetic resonance spectrum (8).

(3) *Monolayers.* Phospholipid molecules possess both hydrophobic and hydrophilic properties and are oriented at a water surface, forming monolayers. For many years, monolayer studies of phospholipids have been conducted. Usually this work has been performed with natural phospholipid mixtures and, in the vast majority of cases, with egg yolk phosphatidylcholine. In recent years a few studies have been made with pure synthetic phospholipids (9). These have shown that, at room temperature, the saturated phospholipids exhibit monolayers which are more condensed than those of unsaturated phospholipids containing *cis* double bonds, i.e., the saturated phospholipids occupy less area at low surface pressures than the corresponding unsaturated compounds. The monolayer behavior of the phospholipids reflects their transition temperatures (10). Thus, at room temperature, a phospholipid which has a high transition temperature exhibits a condensed film; a phospholipid having a lower transition temperature exhibits an expanded film or greater area per molecule (11). This is illustrated in Fig. 2.

(4) *Phase polymorphism.* Phospholipids in the presence of water can also form different liquid crystalline phases. In some cases, as the concentration of water varies, transitions from lamellar to hexagonal phases occur. These transitions have been fully discussed by Luzzati and Husson (12).

In our laboratory recent studies on pure

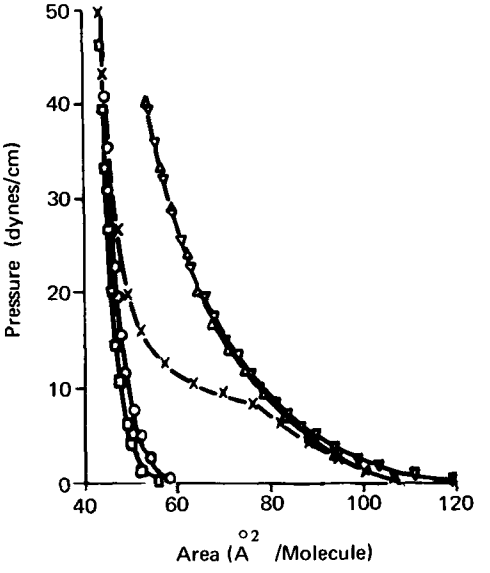

FIG. 2. Pressure area curves for saturated lecithins on aqueous 0.1 M NaCl solution at 22 C: □, dibehenoyl (C_{22}); ○, distearoyl (C_{18}); x, dipalmitoyl (C_{16}); △, dimyristoyl (C_{14}); ▽, dicapryl (C_{10}).

phosphatidyl-cholines in water have shown that over a wide range of concentrations, lamellar/hexagonal transition does not occur. The presence of impurities, such as ions, however, can have an appreciable effect upon the amount of water taken up by the lipid (7).

It is interesting that in water lecithins of different chain lengths adopt a lamellar or bilayer type structure over a wide range of concentrations. At first sight this may appear to lend some support to the idea that a natural membrane may be built up as a bilayer. This, however, need not necessarily be the case. The influence of the protein on the resultant structure may be quite considerable, and the organization of the membrane will then depend on the mode of interaction of the lipid and protein.

(5) *Bilayers.* Recently great interest in the formation of phospholipid bilayer systems has developed (13). The transition temperatures of phospholipids are also relevant to the production of model bilayers which are analogous to cell membranes. When a suitable phospholipid is heated above its transition temperature, satisfactory bilayers can be made. We have shown recently that these bilayers behave optically like a positive uniaxial crystal with the optical axis perpendicular to the plane of the film (14).

Using calorimetric and monolayer techniques, we are presently studying the ways in which lipids of different chain lengths and

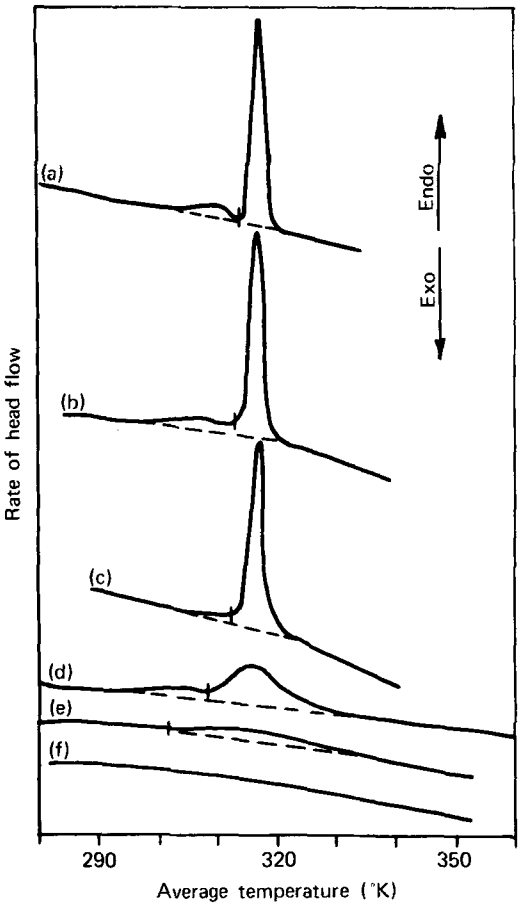

FIG. 3. DSC curves of 50 wt % dispersions in water of 1,2-dipalmitoyl-L-lecithin/ cholesterol mixtures containing (a) 0.0 moles %; (b) 5.0 moles %; (c) 12.5 moles %; (d) 20.0 moles %; (e) 32.0 moles %; and (f) 50.0 moles % of cholesterol.

degrees of unsaturation interact with each other and affect transition temperatures.

Phospholipid - Cholesterol Interactions

Biological aspects. An interaction which may be of considerable biological importance is that of cholesterol with phospholipids. Cholesterol occurs in many membranes, particularly in the myelin sheath and in red blood cell membranes, but its precise arrangement and functions are not understood. The biochemical importance of the solubilization of cholesterol by phospholipid and the vehicular aspects have often been discussed (15).

Monolayer studies. As early as 1925 Leathes (16) showed that cholesterol, when mixed with certain phospholipids in monomolecular films on water, caused a diminution of the area occupied by the fatty acids. Studies of this condensing effect of cholesterol have continued to the present day using monolayer techniques. Despite the many investigations, the interpretation of the effect is still uncertain. Monolayer studies of natural phospholipids, such as egg yolk lecithin, or pure phospholipids containing *cis* unsaturated double bonds, have shown that a film of a more condensed type is produced when cholesterol is present. This has led to a variety of models and discussions of the interaction between phospholipids and cholesterol.

It has been suggested that the presence of a *cis* double bond in the 9:10 position of an acyl moiety of a phospholipid is ideal for combination with a cholesterol molecule (17). It has also been stated that short chain saturated phospholipids which give expanded monolayers nevertheless do not interact with cholesterol (18).

Recent work in our laboratory has indicated that an apparent interaction between cholesterol and phospholipids occurs even when the double bond is not in the 9:10 position, if the monomolecular film of the phospholipid is expanded. Thus, phospholipids containing chains with a double bond in the 6:7 position, or the 10:11 position, also show a condensation effect. Monolayers of the saturated phospholipids usually are condensed and there is no further condensing effect in the presence of cholesterol. However, a condensing effect is found with saturated phospholipids when heated above room temperature. Phospholipids containing a single *trans* double bond have little, if any, condensing effect, although this is not the case with dielaidoyl phospholipids (19). Thus, the presence of a kink in the molecule due to a *cis* double bond at the 9:10 position is *not* a necessary condition for condensation.

Recently studies have been made of bulk phospholipid-cholesterol systems using the DSC technique (20). In Fig. 3 are the DSC curves between 7 and 87 C for a series of dipalmitoyl lecithin-cholesterol mixtures, each containing 50% by weight of water and varying ratios of lecithin to cholesterol. As the concentration of cholesterol increases, the main endothermic transition remains sharp, and a small peak at 35 C disappears. This is followed by a profound change in which the main transition becomes broad and decreases in area. When the concentration reaches 50 mole percent of cholesterol, no endothermic peak is observable. The variations in the transition temperatures and the heats of transition are shown in Fig. 3.

Cholesterol disrupts the ordered array of aliphatic chains in the gel and, when cholesterol and lecithin molecules are present in equimolar proportions, all the chains are in a "fluid" condition.

Interactions with Metal Ions

Some phospholipids, e.g., phosphatidylserines and phosphatidylethanolamines, have a high affinity for Ca^{++} and Mg^{++} and this is related to interactions with their polar groups. We have recently studied the interaction of Ca^{++} with phosphatidylserine and monophosphatidylinositol monolayers (21). We have also used electron spin resonance (ESR) spectroscopy to study the interaction of Mn^{++} with phosphatidylserine dispersions and the competition between ATP and the phospholipid (22).

Relevance to Cell Membranes

The transition temperatures of phospholipids may also be relevant to cell membrane structure. The fluidity of membranes may be related to the transition temperatures of their components. Thus membranes which contain phospholipids having little unsaturation will have less fluidity than those having phospholipids with much unsaturation. This control of fluidity may then be related to diffusional characteristics of molecules passing into and out of the cell.

In line with this idea it is interesting that poikilothermic organisms, which alter their body temperature to correspond to their environment, apparently alter the unsaturation in their phospholipids (23). The alteration may provide a more or less constant fluidity of the membrane structure. At high temperatures the chains are more saturated; at low temperatures, more unsaturated.

Some cell membranes contain compounds having saturated branched chains. In this case the dispersion forces, and hence the transition temperatures, are reduced because of the difficulties in packing of branched chains.

It would be interesting to know whether thermal transitions occur with natural membranes similar to those observed with the phospholipids themselves, and whether the lipids present in a cell membrane always have a lower transition temperature than the particular environmental temperature.

Our studies with myelin isolated from white matter of ox brain (24) show that:

(1) With wet myelin, thermal transitions are not detectable. In this case the cholesterol and other lipids appear to be organized in a single phase. The organization of cholesterol in the membrane appears to prevent the lipids from crystallizing.

(2) To maintain the organization of the lipid in myelin a critical amount of water appears to be required. This water is unfreezable at 0 C and may correspond to "bound" water.

(3) On drying myelin the cholesterol and other lipid crystallize and precipitate. Endothermic transitions associated with the cholesterol and other lipid can then be observed.

(4) The total lipid extract in water does not show a detectable endothermic transition but the cholesterol-free lipid does. In the absence of cholesterol, part of the myelin lipid is crystalline at body temperature.

Other membranes are being examined to determine whether they exhibit similar properties.

LIPID-PROTEIN INTERACTIONS

In our present state of knowledge, there is a danger of oversimplification in attempting to explain lipid-protein interactions, whereas in reality explanation in different cases may be complicated by certain variables.

First, although lipids are generally considered to be related to fatty acids structurally, or metabolically, or in physical properties, the term "lipids," in fact, includes a wide variety of molecular structures. In addition to the classical hydrocarbon or fatty acid antecedents, lipids as now known may contain one or more of a variety of functional groups, among which may be various acidic, basic, and carbohydrate components. In some cases the functional groups give rise to positively or negatively charged regions in lipid molecules, and lipids with zwitterion-type structures may occur. Metal ions are known to be intimately involved in some lipid structures.

Second, there is of course a considerable variety of protein structures that may be involved in lipid-protein interactions. When one considers that interaction of any given lipid and given protein may also vary depending on other materials present, and on pH, ionic strength, temperature, and other variables, it is evident that there may be a variety of ways in which lipids and proteins can interact.

Binding Forces

Some of the binding forces that may be involved in interactions of lipids and proteins are as follows: (a) covalent binding, (b) electrostatic binding, (c) polarization interaction, (d) dispersion interaction, and (e) hydrophobic binding. Other types of binding, in some cases, are conceivable. On the basis of present evidence, it appears probable that electrostatic and hydrophobic binding, and metal ion participation are especially important in the binding of lipid-protein structures.

It is important to note that, in many studies of phospholipid-protein complexes, the phospholipid is dispersed by sonication. Often, phospholipases, which hardly attack coarse

lecithin dispersions, hydrolyze sonicated lecithins at an appreciable rate (25,26). The apoprotein of the mitochondrial enzyme β-hydroxybutyrate dehydrogenase does not react with coarse lecithin dispersions, but does so when the lecithin has been sonicated. Sonication of the lipid breaks up the aggregates to produce small particles which are, however, considered to retain a lamellar type structure (27). We have studied the effect of sonication on aqueous phospholipid dispersions using X-ray analysis, NMR spectroscopy, and electron microscopy, and have confirmed that a breakdown in size occurs from large particles (0.5 to 20 μ) to small particles with diameters less than 1,000 Å (28).

To summarize lipid-protein interactions, it may be said that if lipids and proteins are brought together in water, various consequences can develop, which are dependent also upon the free energy of the products. There are: (a) no interaction and no complex formation, i.e., both lipid and protein retain their original configurations; (b) interaction, with the lipid losing all, or part, of its configuration, i.e., a lipid phase change, but with the protein retaining its configuration; (c) interaction, with the protein losing all, or part, of its configuration, but with the lipid retaining its configuration; (d) interaction, with both lipid and protein losing all, or part, of their original configurations; (e) interaction, with both lipid and protein retaining (largely) their original configurations.

As an example of (b), charged groups on the protein may be neutralized by a few lipid molecules with the protein retaining (largely) its original configuration. As an example of (c), it is conceivable that a protein may unfold its amino acids at the lipid surface, with the lipid configuration remaining largely unchanged. In (d) a gross rearrangement of the long chain moieties of the lipid is conceivable so that they are within the hydrophobic central region of the protein, i.e., associated with the non-polar amino acids; polar groups of both lipid and protein would be on the outside of the complex. In (e) a small interaction might occur involving a sheet of lipid and protein in which both largely retain their original configuration.

From the foregoing it is apparent that physical methods which give information about the shape and size of lipoprotein complexes, and about changes of lipid or protein configurations, should be valuable in studying the nature of lipid-protein complexes. A description of some such methods follows.

X-ray Studies

Studies of the model lipoprotein complexes formed with cytochrome C and basic proteins and serum lipoproteins using conventional X-ray diffraction techniques and small angle X-ray scattering can provide useful information. In particular, small angle X-ray scattering, a technique used successfully to derive size, shape, molecular weight, hydration properties, etc., of proteins in solution, may prove fruitful. In our laboratory (29) we have obtained scattering curves for some of the iso-octane-soluble complexes of cytochrome C with phospholipids. From plots, log τ *versus* h^2, we have derived radii of gyration of the scattering particles, indicating that the complexes are not simple monomers but are aggregated, and we have also shown that at least two complexes exist within the concentration range studied. Further physical studies on this and related model lipoprotein complexes will provide information on the interaction between lipids and proteins in model systems.

Magnetic Resonance Techniques

Magnetic resonance spectroscopy appears to have potential for the study of natural and synthetic lipoprotein complexes. This includes both nuclear magnetic resonance and electron spin resonance spectroscopy.

Nuclear magnetic resonance (NMR) spectroscopy. The basis of NMR spectroscopy is that some nuclei (H^1 and P^{31}) behave like elementary bar magnets and can be lined up in a magnetic field. The absorption of radiation $h\gamma$ can flip the magnets from one energy level to another corresponding to the relation $h\gamma = {}^2\mu H_o$ where H_o is the magnetic field applied and μ is the magnetic moment of the nucleus. The energy separation corresponds to radio frequency radiation. Since only some nuclei exhibit this property, they can be studied, while other nuclei in the molecules remain transparent.

With solids, resonance occurs over a wide field due to dipole-dipole effects of the magnetic units acting on each other. Liquids give a narrow line because the resonance magnetic effects are cancelled out by molecular motion. With good magnetic homogeneity, liquids and solutions show a number of lines related to the various groups in the molecule. Hence, a lecithin molecule gives separate lines associated with the resonance absorption of radiation due to the protons in $[CH_2]_n$, $N(CH_3)_3$, and HC=CH groups, etc. (30). Thus interaction of lipid and protein can be studied by comparing the interaction spectrum with the separate spectra of the lipid and protein.

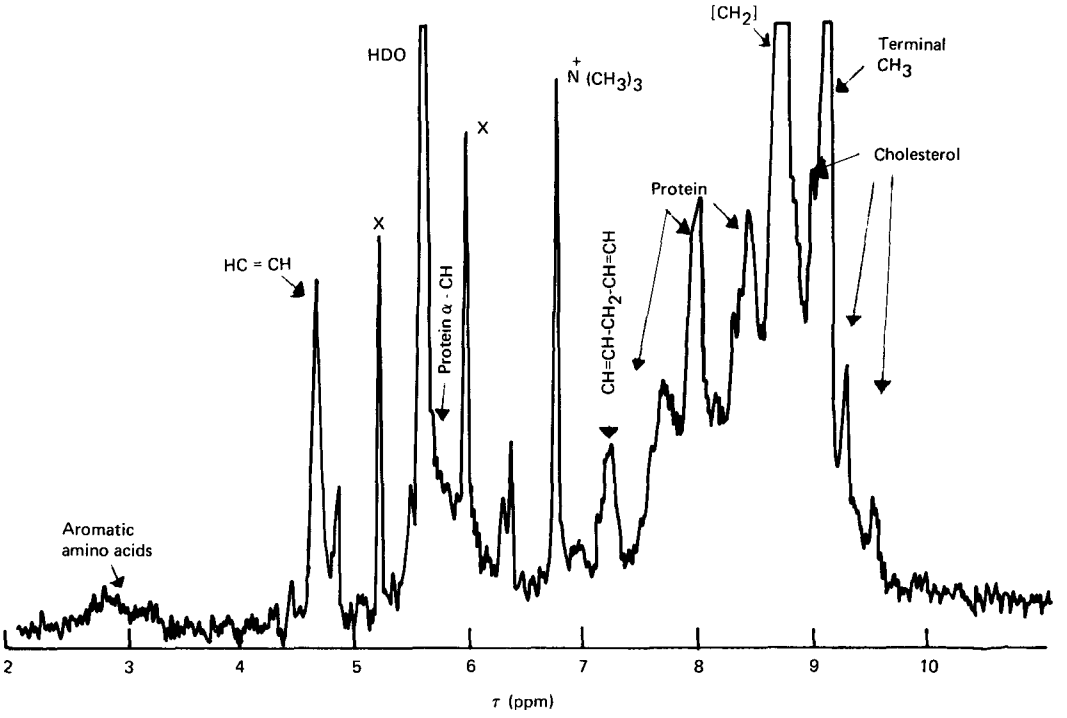

FIG. 4. The 220 Mc/s proton magnetic resonance spectrum of human serum α-lipoprotein (sub-fraction HDL_2) in D_2O at 50 C. Peaks marked x are spinning side bands.

We have studied the bulk mesomorphic behavior of phospholipids (31), phospholipid dispersions in water (32) and water solutions of bile salts (33). More recently we have studied serum lipoproteins (34), and the lipid-protein interactions in aqueous dispersions of fragments of erythrocyte membranes (35).

The high density α-lipoprotein (HDL), one of the major lipoprotein classes of human serum, contains about 50% lipid by weight and can be separated with a high degree of purity from other serum lipoproteins (36). Ultracentrifugation provides a means for separating HDL into two major classes, HDL_2 (d = 1.125) and HDL_3 (d = 1.125 - 1.21).

The water-soluble high density α-lipoproteins of serum can be freed of lipids by careful treatment with organic solvents to yield a water-soluble apoprotein that has been characterized, chemically and immunologically, in some detail. It binds lipids avidly, particularly phospholipids, to generate water-soluble reconstituted lipoproteins (37).

Recent developments have demonstrated the advantages of high resolving power of the 220 Mc/s NMR spectrometer for the study of protein conformation. We have used this instrument to study the proton resonance spectra of lipoproteins and other systems.

The first prominent feature which is apparent in the 220 Mc/s spectrum of HDL_2 lipoprotein shown in Fig. 4 are the signals associated with the lipid material. These can be clearly seen including those of various functional groups that contain chemically shifted protons in the phospholipid moiety, such as in the $N(CH_3)_3$, $(CH_2)_n$, terminal CH_3, and HC=CH groups. This is of particular interest because these are the signals which one observes when a phospholipid (e.g., lecithin) is dissolved in an organic solvent such as chloroform (30). In contrast, high resolution signals cannot be obtained if one disperses phospholipid alone in water, without sonication (32). Thus, the first conclusion that one can draw about HDL lipoproteins, both HDl_2 and HDL_3, is that the lipid material present appears to be in a magnetically isotropic environment with considerable molecular freedom, such as exists when the lipid is dissolved in organic solvents. This is unlike what one finds with erythrocyte membranes (35).

A number of differences were observed when the NMR spectra of lipoproteins were examined at high temperatures (50 C). The signals associated with the aromatic amino acids of the protein are not increased very much in intensity, but the signals associated with the

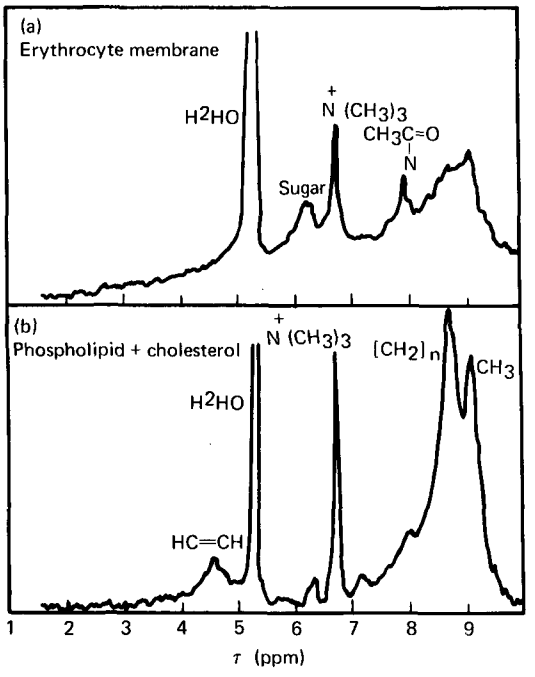

FIG. 5. The proton magnetic resonance spectra at 33.4 C of a 5% (w/v) dispersion in D_2O of (a) erythrocyte membrane fragments and (b) total equivalent lipid (phospholipid + cholesterol). (64 scans each)

lipid moiety become sharper. The signal associated with the $N(CH_3)_3$ group is particularly sharp, of the order of ~6 Mc/s Sharp signals are also observed on each side of the terminal methyl signal at 9.1 τ. At first it was thought that these signals might be due to ring shifted methyl signals, as observed in the spectrum of lysozyme (38). It became apparent, on comparison of the positions of these signals with those of several cholesterol esters, that they were due to the methyl groups associated with the ring system of the cholesterol esters in the lipoprotein. This suggested that, at low temperature, the ring system associated with the cholesterol moiety was in an anisotropic condition, or was slightly restricted in its molecular freedom and that, on increasing the temperature, there was greater mobility of the lipid chains and also of some of the amino acids of the protein moiety. The greater freedom of the amino acids appears to be such as to allow greater mobility of the cholesterol ring structure.

After reconstitution of the apoprotein with phospholipid, an NMR spectrum similar to that of the original lipoprotein was observed. Resolution in the spectrum of the reconstituted lipoprotein at low temperature (13 C) was more like that of the high resolution spectrum of the original lipoprotein at 50 C, as though the reconstituted lipoprotein had a less compact structure. It may be that the other lipid material, e.g., the cholesterol esters, are important in determining the compactness of the structure. No particular differential broadening effect is observed with any signals associated with the phospholipid as a result of phospholipid-protein interaction.

The fact that it is possible to obtain high resolution NMR spectra from phospholipid dispersed in aqueous systems led to the idea that biological membranes themselves could be examined using this technique. There is considerable molecular motion of phospholipid in the presence of water so that one might expect that an NMR signal could be obtained from the lipids in the membrane itself.

The first experiments carried out (35) involved examination of the proton magnetic resonance (PMR) spectra of membranes, in particular of the erythrocyte membranes or ghosts, when dispersed in D_2O. These studies indicated that useful information can be obtained. PMR spectra of erythrocyte membranes after sonication are shown in Fig. 5. Peaks associated with some of the functional groups of compounds in the membrane can be clearly observed. Thus there is a peak at 6.8 ppm which arises from the 9 protons present in the choline group of the phosphatidylcholines and sphingomyelins present in the membrane. Peaks are also observed which have been assigned to the protons present in sugar groupings (6.3 ppm), and also to sialic acid (7.3 ppm) which is known to be associated with the erythrocyte ghost material. The PMR spectra of ox brain ganglioside and N-acetyl neuraminic acid give peaks near these positions and aid the assignment.

Interestingly, the considerable number of protons present in the long chains of the phospholipids do not give rise to a strong peak. A broad hump occurs in the 8.8 ppm region rather than a sharp peak. It is similar to that observed when phospholipid-cholesterol interactions occur. A signal at 4.7 ppm which would be due to protons in HC=CH in the chains is not apparent. These results can be contrasted with those obtained with serum lipoproteins. It appears that the lipid-protein interaction provides a more compact structure in the membrane.

Addition of a molecule, such as sodium desoxycholate, causes a progressive increase in the alkyl chain signal, and it appears that the sodium desoxycholate has weakened the lipid-protein interaction and released lipid from the membrane.

Our interpretation of the spectral changes

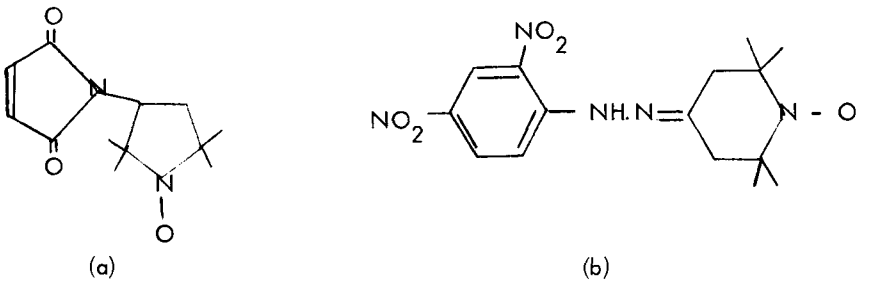

FIG. 6. Two spin labels used in electron spin resonance experiments (a) N-1-oxyl-2,2,5,5-tetramethyl-pyrrolidinyl) -maleimide (b) 2,4-dinitrophenylhydrazone of 2,2,6,6-tetramethyl-4-piperidone.

observed with sodium desoxycholate is that it liberates lipids from the protein and then forms a mixed micelle with these lipids. Bile salts and lipids are known to form mixed micelles (39). In such micelles, molecular motion of the desoxycholate proton groupings may be inhibited but molecular motion of the lipid is quite considerable.

The spectrum of the membrane co-dispersed with lysolecithin showed features which are consistent with the formation of an additive complex with the membrane material. A complex micelle formation has been reported in the case of myelin lipoprotein solubilized by lysolecithin (40).

Spectra of erythrocyte membranes co-dispersed with increasing concentrations of sodium dodecyl sulphate (41) show interesting changes. At concentrations greater than 0.4 mg/mg of membrane protein, the signal for $(CH_2)_n$ at 8.7 ppm begins to narrow and the peak at 4.7 ppm becomes apparent, which is due to protons of the CH=CH group. At a concentration of 0.8 mg sodium dodecyl sulphate/mg membrane protein, a new peak occurs at 2.4 - 2.6 ppm due to amino acids of the membrane protein. This peak is not observed in the spectra of erythrocyte membranes treated with comparable concentrations of sodium desoxycholate. However, narrowing of the chain signal and appearance of peaks for a CH=CH group show a similar dependence on concentration.

We have also examined myelin and the envelopes of *Halobacterium halobium*.* Brown and Shorey (42) have shown that cell envelopes of *H. halobium* are stable in high salt concentrations (4 to 5 M NaCl), but dissolve rapidly as the salt solution in which they are suspended is diluted. In distilled water they give lipoprotein complexes. The intact cells are considered to be bounded by a single membrane, devoid of cell walls. Some evidence, however, has been given recently (43) to suggest the existence of a labile cell wall.

The PMR spectrum of the envelopes in a 4 M NaCl solution in D_2O is almost featureless, exhibiting only very weak and broad absorption between 8 and 9 ppm. However, when disaggregation of these envelopes occurs by dilution with D_2O (to salt concentrations less than 0.8 M), a spectrum with well resolved proton resonance signals is observed.

We conclude that disaggregation of the envelopes at low ionic strength produces increased freedom for some amino acid protons of the protein. These assignments are consistent with the evidence (44) that only 20% of the lipoprotein of the envelopes is lipid and that, upon dilution, more groups of the envelope protein become available for titration.

Electron spin resonance (ESR). This magnetic resonance technique also has potential for the study of lipoprotein complexes and membranes. In this case an unpaired electron is required in the molecule before a signal can be observed. Since this does not usually occur naturally with lipids and proteins, spin labels containing an unpaired electron are prepared. The structures of two spin labels are shown in Fig. 6. These can be attached either to the protein or to the lipid, and the ESR spectrum of the spin label studied before and after complex formation. Recent studies (45) have been published of the cytochrome C complex where the protein was labelled with N-(1-oxyl-2,2,5,5-tetramethyl-pyrrolidinyl)-maleimide. Recently we have also studied spin labelled cell membranes (46).

CONCLUSIONS AND FUTURE STUDIES

Physical techniques have given useful and important information about the behavior of phospholipids, and the interactions which these molecules undergo with water, cholesterol, and protein. Further studies should yield additional knowledge about these interactions, thereby providing useful guides to an understanding of

*The study of *H. halobium* is being carried out in collaboration with Miss J. Cullen and a full account of our studies will appear elsewhere.

the behavior of emulsifiers, and of the structures of lipoproteins and cell membranes. Information about the mode of interaction of detergents and drug molecules with membranes may arise from similar studies. Combined with studies of model membranes, the way in which excitable characteristics occur in cell membranes may also be revealed.

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Studies on Positional Specificity of the Castor Bean Acid Lipase

ROBERT L. ORY, JAMES KISER and **PAUL A. PRADEL**
Southern Regional Research Laboratory[1] New Orleans, Louisiana 70119

ABSTRACT

The acid lipase of castor bean endosperm catalyzed the hydrolysis of fatty acids from the 1 and 3 positions of synthetic glycerides immediately after achieving proper reaction conditions, but fatty acids from the 2 position were not detected in the reaction products until 7 to 10 min later. Results obtained with 2,3-butane dioleate, n-hexyl oleate and 2-hexyl oleate showed that the castor lipase does not cleave secondary ester linkages. These findings suggest that the acid lipase of the castor bean may catalyze hydrolysis of fatty acids from the 1 and 3 positions of triglycerides only; the steady appearance of 2 position fatty acids in the reaction products during lipolysis is probably the result of an apparent isomerization reaction.

INTRODUCTION

The specificity of the castor bean lipase (EC 3.1.1.3) for fatty acid chain length in single acid triglycerides was reported several years ago(1). A study on positional specificity of the enzyme was delayed for lack of suitable substrates. Savory et al. (2), in experiments using olive oil as substrate, reported that the castor lipase was not specific for position; that inner fatty acids of triglycerides were cleaved as well as outer ones. In our laboratory using cottonseed oil as substrate, we reported that hydrolysis in the castor lipase system was rapid and complete with no evidence for reversal of hydrolysis (3). The normal assumption, based on such data, was that the castor lipase was nonspecific for position. However, rapid and complete appearance of the fatty acid from the 2 position does not necessarily mean release from that position by the lipase. Isomerization of 2-monoglyceride to 1-monoglyceride is possible under certain conditions of pH, temperature and time of reaction (4).

For these investigations, we have controlled these conditions and have used synthetic substrates in attempts to limit or overcome the migration of the inner fatty acid to the primary position. Triglycerides having a different fatty acid in the 2 position were used on one phase of the work. In the second phase, secondary esters having no primary hydroxyl groups were employed as substrates to prevent isomerization from 2 to 1 positions. These latter type esters having been employed by other investigators of lipase positional specificity (5,6). The results of our investigations are described.

EXPERIMENTAL PROCEDURES

Seed

Castor beans, *Ricinus communis*, Baker 296 variety, were a gift from W. E. Domingo and D. S. Bolley, Baker Castor Oil Company.

Materials

All inorganic salts and organic solvents were analytical grade materials purchased from commercial suppliers. Cottonseed oil used as a substrate was commercial Wesson Oil. 1,3-Dipalmitin, 1,3-diolein, and boron triflouride were purchased from Applied Science Laboratories; palmitoyl and oleoyl chlorides from Universal Oil Products, Chemical Division; and silica gel G from Brinkmann Instruments Company. 2,3-Butane dioleate, n-hexyl oleate, and 2-hexyl oleate were generous gifts from Dr. Fred Mattson, Proctor & Gamble Company.

Methods

Preparation of Lipase. The acid lipase was prepared by sequential extraction of castor beans with pH 7.5 phosphate-cysteine-EDTA buffer, ether, and salt solutions as described earlier (7). For the tests involving 2,3-butane dioleate as substrate, the lipase employed was a suspension in *tris* buffer, pH 7.5, containing 0.05 M cysteine. Our early work (1,3) showed that this enzyme was SH-sensitive and was quite stable in dry form. However, to insure maximum activity the enzyme was incubated overnight under nitrogen in the refrigerator in the above buffer.

Preparation of Triglycerides. The specific triglycerides 1,3-dipalmitoyl, 2-oleoyl glycerol (POP), and 1,3-dioleoyl, 2-palmitoyl glycerol (OPO), were synthesized by acylation of the 1,3-diglycerides with excess acyl chloride in chloroform and quinoline (8). The final products were repeatedly washed with bicarbonate until free of fatty acids as detected by thin

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

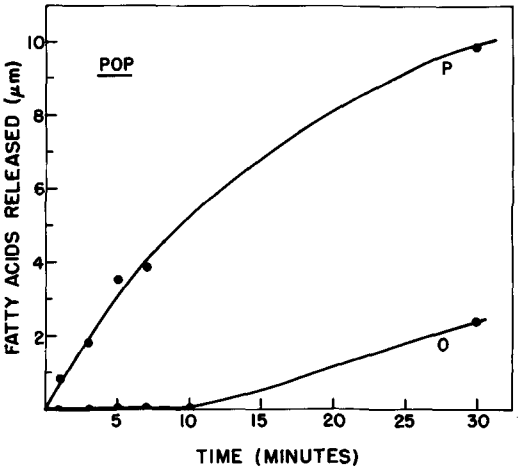

FIG. 1. Release of fatty acids from POP catalyzed by castor bean acid lipase; 146 mg POP used in this experiment; other conditions as described in "Procedure for Lipolysis Tests."

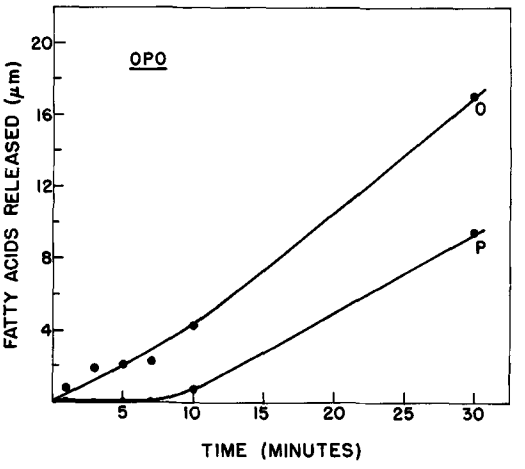

FIG. 2. Release of fatty acids from OPO catalyzed by castor bean acid lipase; 374 mg OPO used in this experiment; other conditions as described in "Procedure for Lipolysis Tests."

layer chromatography (TLC) on silica gel impregnated with silver nitrate (9).

Measurement of Lipolysis. Optimum conditions of pH and time of reaction were developed using cottonseed oil as substrate. Lipase activity on this and on butane dioleate was measured by titrating the fatty acids released from the substrate with 0.1 M sodium hydroxide in a Radiometer pH-Stat as described earlier (7). Fatty acids hydrolyzed from the synthetic triglycerides at increasing times of reaction were analyzed by gas liquid chromatography (GLC) in a Model 1520 Varian Aerograph.

Procedure for Lipolysis Tests. For the triglyceride experiments 10 mg of dry lipase preparation, 100 to 300 mg of POP or OPO, and water were honogenized in a Potter-Elvehjem glass homogenizer for 1 min. The tube was rinsed twice with water and the pH was lowered to 4.2 with 1 N acetic acid; total volume of the reaction was 6 ml. At various times 0.5 ml aliquots were removed by pipette directly into 20 ml of diethyl ether and 10 ml of sodium bicarbonate to stop the reaction. The ether layer was extracted once more with sodium bicarbonate to remove the hydrolyzed fatty acids from the unhydrolyzed glycerides. The combined bicarbonate solutions were acidified with sulfuric acid to pH 4-5 and again extracted with two 10 ml portions of ether to isolate the fatty acids. Ether extracts were dried over anhydrous sodium sulfate and the ether removed by stripping with nitrogen gas under an incandescent lamp. Methyl esters of the fatty acids were prepared with boron triflouride in methanol (10). The methyl esters of the palmitic and oleic acids released in the above reactions by the castor lipase were then analyzed by GLC.

Procedure for GLC Analyses. Standard curves for quantitative analysis of the results were prepared using methyl esters of authentic palmitic and oleic acids. The GLC columns were ¼ in. by 2 ft aluminum tubing packed with 100/120 mesh Chromasorb W coated with 3% SE 30. Injector temperature was 227 C; the column was 177 C, and thermal detector was 207 C; MA was 249.

Samples of 1-10λ were injected to obtain peaks of good size and each was done in triplicate. The areas of the peaks were determined by cutting out the chromatographic peaks, weighing them individually on a Mettler analytical balance, and using the average values of each three weighings for subsequent plotting of data.

RESULTS AND DISCUSSION

Mattson and Volpenhein (4) studied the isomerization of 2- to 1-monoglyceride under varied conditions of pH, temperature and reaction time. The rate of isomerization of 2- to 1-monolein dropped considerably within 30 min while changing the pH from 8.6 to 6.0, but they stated that the use of more acidic solutions would undoubtedly result in higher rates. In order to minimize any isomerization of the 2 position fatty acids in these experiments on POP and OPO, the reactions were limited to 30 min and were conducted at room temperature and at the optimum pH for this enzyme, 4.2 (1). It was felt that longer reaction times at pH 4.2 would enhance any isomerization which might occur in this system.

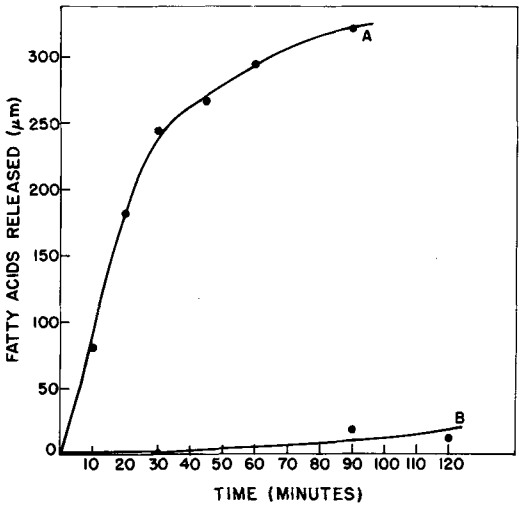

FIG. 3. Activity of castor bean lipase on cottonseed oil and 2,3-butane dioleate. Curve A, 150 mg cottonseed oil used as substrate per test; curve B, 46-50 mg 2,3-butane dioleate used per test; other conditions as described in "Measurement of Lipolysis."

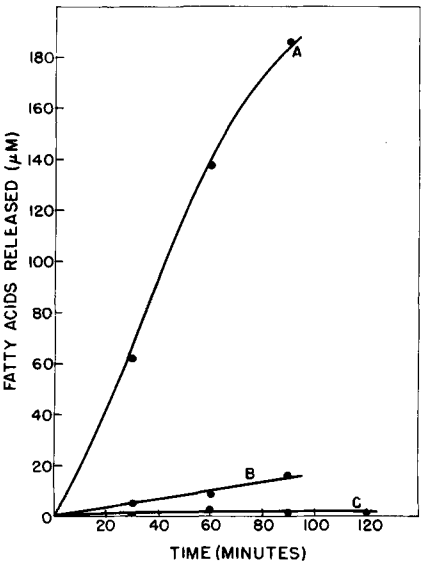

FIG. 4. Activity of castor bean lipase on cottonseed oil, n-, and 2-hexyl oleates. Curve A, 150 mg cottonseed oil used as substrate per test; Curve B, 75-80 mg n-hexyl oleate used per test; Curve C, 75-80 mg 2-hexyl oleate per test; other conditions as described in "Measurement of Lipolysis."

The results of experiments with POP as substrate (Fig. 1) show that the primary esters are cleaved immediately but that the inner fatty acid is not detected in the reaction products until after 10 min. Similar results were obtained with OPO as substrate (Fig. 2), though larger quantities of triglycerides were initially present in these experiments.

These results may be interpreted in several ways. If this lipase is nonspecific, similar to the pancreatic enzyme[2] reported by Mattson and Volpenhein (6), then one might conclude that both primary fatty acids are removed before the inner ester bond is broken; the time for this reaction to take place under these conditions being about 10 min. However, if reaction conditions are promoting isomerization, then the lipase is catalyzing hydrolysis of the newly-formed 1-monoglyceride as quickly as it is formed; the time for appearance of quantities of the inner fatty acid sufficient for detection being about 10 min. There is the added possibility of an isomerase system existing in the seed, but there is no evidence to support this at present.

With OPO substrate it may be pointed out that one palmitate would be released for every two oleates if the enzyme is nonspecific for position. This does occur but only after 20 min have elapsed, providing evidence in favor of a prior isomerization of 2- to 1-monoglyceride before the inner fatty acid is cleaved.

In order to determine if the castor lipase is capable of hydrolyzing secondary ester bonds of a substrate in which the primary carbon atoms have no free hydroxyl to which the inner fatty acids can migrate, 2,3-butane dioleate was employed as a substrate. Optimum conditions for lipolysis were determined using cottonseed oil as substrate (Fig. 3, Curve A) and as the reference material. The results in Fig. 3 show that hydrolysis of secondary fatty acid ester bonds in 2,3-butane dioleate (Curve B) catalyzed by the castor lipase is extremely slow, if it does indeed occur. Only 11-17 μmoles of oleic acid (theoretical yield, 164) were released after 2 hr of reaction. This small amount of activity on the artificial substrate might be compared to results obtained by Mattson and Volpenhein (6) with pancreatic lipase. They also found that hydrolysis of the secondary ester group by a treated enzyme, or by an untreated enzyme in the absence of bile salts, was so slow that a measurable initial rate could not be obtained on the pH- stat.

The ability of the castor lipase to catalyze hydrolysis of secondary esters was further tested by comparing normal and secondary hexyl oleates and cottonseed oil as substrates (Fig. 4). The results using 2-hexyl oleate (Curve

[2]The nonspecific pancreatic lipase has an absolute requirement for bile salts, but no emulsifiers were added in these experiments since a lipid cofactor separated from the castor lipase (11) has been found to act as a natural emulsifier (12).

C) confirm those obtained with the butane dioleate substrate; this lipase does not catalyze hydrolysis or secondary esters. The small amount of hydrolysis of n-hexyl oleate (Curve B) seems to be related to this enzyme's low activity on monohydric alcohol esters. Earlier studies (1) showed that the lipase was relatively inactive on methyl and butyl ricinoleates.

The logical conclusion drawn from these results is that the castor bean lipase, contrary to earlier beliefs (2,3), seems to be positional specific. It appears that the lipase system in this seed functions at an acid pH in which isomerization of 2-monoglycerides to 1-monoglycerides is favored. Under such conditions lipolysis of storage oil can occur rapidly and completely.

Less work on positional specificity on seed lipases has been done compared to those investigations on animal (4-6) and microbial lipases (13,14). The reasons for this are unknown. It may be that, except for the castor bean, most oilseeds must be germinated and enzyme activity is low. Consequently, very long reaction times are required to obtain sufficient measurable fatty acids. Whatever the reason, the results of these experiments suggest that this seed lipase is similar to the pancreatic lipase with respect to its positional specificity.

ACKNOWLEDGMENTS

G. T. Pittman made the drawings.

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[Received September 16, 1968]

Fatty Acid Desaturase Systems of Hen Liver and Their Inhibition by Cyclopropene Fatty Acids

A. R. JOHNSON, A. C. FOGERTY, JUDITH A. PEARSON, F. S. SHENSTONE and AUDREY M. BERSTEN, CSIRO Division of Food Preservation, Ryde and Biochemistry Department, University of Sydney, N. S. W., Australia

ABSTRACT

Hen liver preparations which desaturate stearic acid at the 9,10 position to form oleic acid have been found to desaturate other saturated fatty acids of carbon chain length from 12 to 20 and 22. The 9,10-monoenoic fatty acid of the same chain length as the substrate fatty acid is the major product formed. Minor amounts of the 10,11- and 11,12-monoenoic acids are also formed. Maximum desaturation occurred with the C_{14} fatty acid substrate and with the fatty acids C_{17} and C_{18}, suggesting the presence of at least two desaturating systems. The cyclopropene fatty acids, sterculic and malvalic acids, inhibited the desaturation of all the fatty acids at the 9,10 position but desaturation at the 10,11 and 11,12 positions was affected only slightly. The effect is not due to inhibition of the primary activating enzyme, the long chain acyl CoA synthetase. Sterculic acid is a more effective inhibitor than either malvalic acid or sterculyl alcohol, probably because these cyclopropene compounds do not block the desaturating site of the enzyme as completely as sterculic acid.

INTRODUCTION

The cyclopropene fatty acids inhibit the system which desaturates stearic acid to oleic acid in hen liver (1,2), in chicks (3,4), in rats (5,6) and in plant tissues (7). In the desaturase system of hen liver, sterculic acid was more effective than malvalic acid in inhibiting the 9,10 desaturation of stearic acid (2). The difference was probably related to the position of the cyclopropene ring in the two inhibitors, sterculic acid having the ring at the 9,10 position and malvalic acid at the 8,9 position. To determine whether differences existed with substrates of different chain lengths the effect of the two cyclopropene fatty acids on the desaturation of saturated fatty acids from C_{12} to C_{20} was investigated. The influence of chain length on the dehydrogenation of saturated fatty acids has recently been studied in plant tissues (8) and in rat liver (Kellerman & Williams, private communication, 1963) (9).

EXPERIMENTAL PROCEDURES

In the initial stages of this work 1-^{14}C-labeled fatty acids of low specific activity, prepared and donated by G. M. Kellerman and R. A. Williams, University of Sydney, were used. Most of the work was carried out using 1-^{14}C-labeled fatty acids obtained from Mallinckrodt Nuclear, Florida, (C_{13}, C_{15}, C_{19}) or from the Radiochemical Centre, Amersham, England (C_{12}, C_{14}, C_{16}, C_{17}, C_{18}, C_{20}). Carrier fatty acids of high purity were obtained from commercial sources and gas radiochromatography or gas-liquid chromatography (GLC) of the methyl esters showed that the purity of the labeled and carrier acids was at least 99%. The cyclopropene fatty acid inhibitor solutions were prepared by saponification of pure methyl sterculate and methyl malvalate (2). Sterculyl alcohol was prepared from methyl sterculate by the method of Kircher (10).

Liver preparations containing soluble enzymes and microsomes of homogenized livers of White Leghorn hens were prepared as previously described (1). The desaturase assay system, total volume 10.1 ml, comprised: 3 ml liver preparation; 3 ml cofactor solution (1 ml 0.1 M adenosine triphosphate; 1 ml 0.5 M lactic acid; 1 ml 0.5 M KH_2PO_4; 2 mg nicotinamide adenine dinucleotide; 1 mg coenzyme A (CoA); all solutions had been adjusted with NaOH to pH 7.4); 2 ml 0.55 mM potassium salt of the substrate fatty acid in 2.5% aqueous bovine serum albumin (BSA, Sigma Chemical Co., Missouri or K & K Laboratories, California); 0.1 ml 0.1 M $MgCl_2$ (added simultaneously with the substrate); and 2 ml 2.5% BSA in which the potassium salts of the cyclopropene fatty acids were dissolved when their effect was being studied. Sterculyl alcohol was solubilized with Tween 80; the latter was shown to have no effect on the hen liver desaturase system.

The assay system was incubated by shaking in flasks for 30 min at 37 C, after flushing out with oxygen. When the effect of the cyclopropene fatty acid inhibitors was being studied the control flasks and those containing inhibitor were pre-incubated for 10 min to allow reaction between the desaturase system and the inhibitor. The substrate fatty acid was then

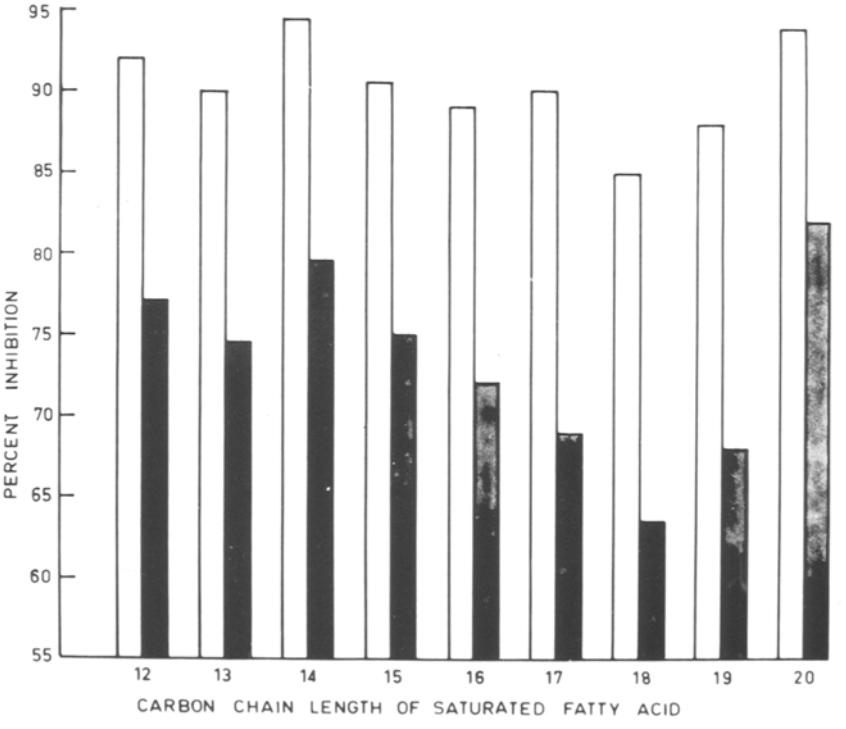

FIG. 1. Desaturation of fatty acids of varying chain length by the desaturase system prepared from each of three hen livers, A, B and C. Dotted line represents mean; least significant difference between means (P = 0.05) = 10.1%.

added and incubation was continued further for 30 min.

After incubation aqueous N KOH solution (5 ml) was added to each flask and the mixture was refluxed for 90 min to saponify the lipid. Fatty acids were isolated, converted to methyl esters and the purified esters were separated into saturated and unsaturated fractions as described previously (2). Radioactivity was determined at each stage in the procedures (2).

The activity of the desaturase system for each substrate was expressed as per cent desaturation at 30 min calculated from the ratio of the count found in the unsaturated methyl ester fraction to the sum of the counts found in the saturated and unsaturated methyl ester fractions.

Radioactive components in the saturated and unsaturated methyl ester fractions were determined by gas radiochromatography. The site of introduction of the double bond into the saturated fatty acid substrates by the hen liver desaturase system was determined by oxidizing the unsaturated fatty acid methyl esters from each substrate, essentially by the procedure of Tinoco and Miljanich (11). The acid products of each oxidation were converted to methyl esters using boron trifluoride-methanol (12) and the esters were separated by chromatography on Florisil (Floridin Co., Florida) containing 7% w/w water. The mono-carboxylic acid methyl esters were eluted with 5% ether-hexane (v/v) and the dicarboxylic acid esters with 20% ether-hexane (v/v). Only the dimethyl esters of the dicarboxylic acids carried the ^{14}C label, and gas radiochromatography of the dimethyl esters was used to determine the labeled components. The amounts of 9,10-, 10,11-, and 11,12-monoenoic fatty acids produced from each substrate were calculated from the per cent desaturation and the distribution of label in the dicarboxylic acids.

The long chain acyl CoA synthetase was isolated from beef heart mitochondria by cholate treatment (9,13). The acyl CoA synthetase remained particulate and the preparation exhibited no desaturase activity (9). The enzyme was assayed by hydroxamate trapping of the activated acyl group (14) and the activity was expressed as μmoles of acylhydroxamate produced per hour. Aqueous solutions of either potassium stearate or potassium sterculate, or both, were added to the complete incubation mixture at 37 C to start the reaction.

TABLE I

Desaturation Products of Fatty Acids of Varying Chain Length by the Desaturase System of Hen Liver in the Presence or Absence of Cyclopropene Fatty Acids[a]

Substrate (0.11 mM)	Type of incubation[b]	9,10-Unsaturates[c,d]	10,11-Unsaturates[c]	11,12-Unsaturates[c]
C12:0	C	110	1.8	3.1
	S	8	0.3	1.0
	M	24	0.5	1.4
C13:0	C	236	3.3	1.1
	S	22	0.4	0.2
	M	66	0.7	1.4
C14:0	C	621	4.4	4.4
	S	29	0.3	1.2
	M	106	0.9	1.4
C15:0	C	174	0.9	0.7
	S	18	1.0	0.5
	M	52	0.7	0.5
C16:0	C	423	3.3	Trace
	S	44	0.8	1.0
	M	117	1.1	1.1
C17:0	C	461	3.3	Trace
	S	45	0.7	1.0
	M	142	0.9	1.1
C18:0	C	521	3.3	1.1
	S	71	1.2	1.2
	M	163	1.4	1.0
C19:0	C	156	1.0	0.3
	S	31	0.5	0.2
	M	66	1.1	0.3
C20:0	C	189	1.1	Trace
	S	11	0.3	0.5
	M	32	0.7	1.0

[a]Millimicromoles of product per μmole of substrate in 30 min.

[b]C, control; S, inhibited by sterculic acid (0.0005 mM); M, inhibited by malvalic acid (0.0005 mM).

[c]Based on dicarboxylic acids formed in oxidation, and on per cent desaturation using results obtained from liver C. (Fig. 1).

[d]May contain small amounts of 7,8 and 8,9 unsaturates.

RESULTS AND DISCUSSION

The per cent desaturation values obtained with desaturase systems from 11 different livers were statistically analyzed and the mean values for the fatty acids C_{14}, C_{16}, C_{17} and C_{18} were 58.2, 45.2, 50.5 and 50.6 respectively (standard error of the means 1.96 on 30 degrees of freedom). Since the ability to desaturate varied considerably from one liver preparation to another the whole range of fatty acid substrates was used on each of three separate hen livers. When this was carried out a common pattern was apparent (Fig. 1) in which the extent of desaturation depended on the chain length of the substrate fatty acids. Maximum desaturation occurred with the C_{14} fatty acid substrate and with the fatty acids C_{17} and C_{18}, suggesting that at least two desaturase systems were present. However, desaturation involves many steps including activation, transfer mechanisms and removal of the product by incorporation into complex lipid; the influence of chain-length may be operative at any of these stages, but it has been shown (9) for rat liver that activation was not rate-limiting in the desaturation of the fatty acids C_{14} to C_{18}.

The major product from each substrate fatty acid was shown to be the monoenoic fatty acid of the same chain length by gas radiochromatography. This product constituted over 90% of the products of desaturation in all cases, whether cyclopropene fatty acid inhibitors were present or not. Trace amounts of other saturated and unsaturated fatty acids were also produced from each substrate, apparently by elongation or shortening of the chain by two-carbon units.

Introduction of the double bond into each fatty acid by the hen liver desaturase system was predominantly at the 9,10 position (Table I) and to a slight extent at the 10,11 and 11,12 positions. After oxidation of the desaturation

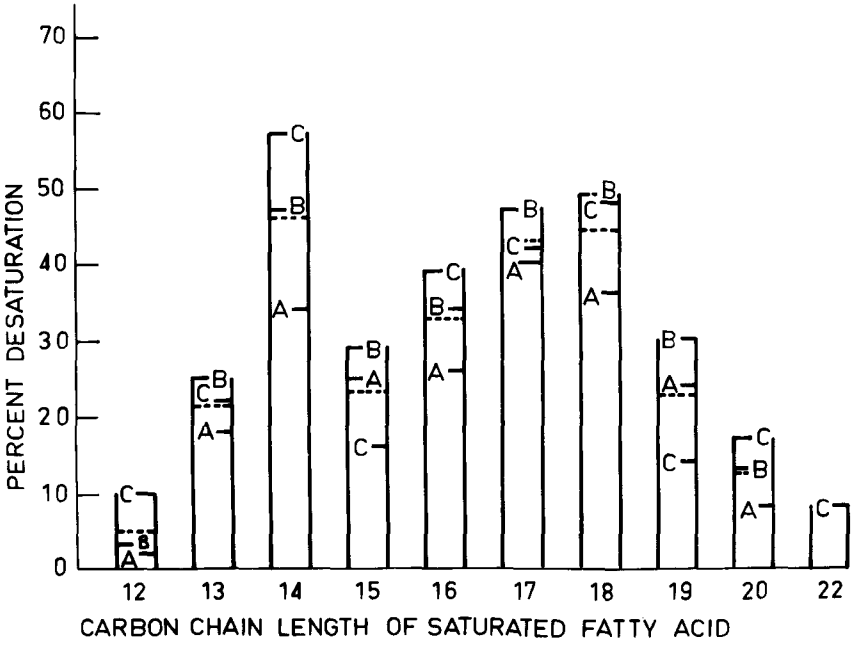

FIG. 2. Mean per cent inhibition of desaturation of fatty acids of varying chain lengths by sterculic and malvalic acids (0.0005 mM) in desaturating systems from two hen livers (A and B, Fig. 1). The open blocks represent sterculic acid and shaded blocks malvalic acid. The per cent inhibition was calculated using the per cent desaturation from the uninhibited systems as 100% conversion.

products the amount of radioactivity in the C_7 and C_8 dicarboxylic acids was variable, but low, and was attributed mainly to over-oxidation.

Since the desaturase system of hen liver desaturates all the saturated acids from C_{12} to C_{20} at the 9,10 position, the site of desaturation must be independent of the number of methylene groups beyond the 9,10 position. Similar results were obtained by Howling, Morris and James (8) for 9,10 desaturation of fatty acids by *Chlorella vulgaris*; these workers conclude that a direct attachment of the saturated fatty acid, or of the CoA ester, or of the acyl carrier protein thiol ester occurs with the desaturase enzyme complex. We came to the same conclusion and envisage a primary attachment involving the carboxyl end of the CoA ester of the substrate, followed by alignment of the 9,10 bond at the desaturating site, which must be separated from the first site of attachment by a distance corresponding to nine carbon atoms.

The cyclopropene fatty acids, sterculic acid and malvalic acid, strongly inhibited desaturation of each substrate by the desaturase system (Table I and Fig. 2). Inhibition of the desaturation of the C_{14} fatty acid by either cyclopropene acid was greater than inhibition of C_{17} and C_{18} desaturation, which again suggests the presence of at least two desaturase systems. The double bond was still introduced predominantly into the 9,10 position (Table I). The 9,10 desaturation was very strongly inhibited by the cyclopropene acids, while 10,11 desaturation was only partially inhibited and 11,12 desaturation was probably not affected. Sterculic acid was a more effective inhibitor than malvalic acid for both 9,10 and 10,11 desaturation.

The initial step in desaturation is activation by an acyl CoA synthetase of the long chain fatty acid to form the acyl CoA derivative. Inhibition of desaturation could perhaps be attributed to inhibition of the acyl CoA synthetase. However, when the activity of the acyl CoA synthetase isolated from beef heart mitochondria was measured with stearic acid, as a substrate, the addition of sterculic acid at levels of one tenth and one hundredth that of the substrate stearic acid (0.5 mM) had no inhibitory effect. This indicates that the fatty acid activating reaction is probably not the rate determining step in the inhibition of the desaturase system, because at these concentrations of sterculic acid the desaturase system of hen liver would be completely inhibited. The rate of activation of sterculic acid by the beef heart acyl CoA synthetase was higher than that of stearic acid, 1.12 μmoles and 0.68 μmoles hydroxamate per hour respectively at 0.5 mM

substrate level. The high activation rate for sterculic acid suggests that sterculyl-CoA, rather than the free acid, may in fact be the actual inhibitor of the desaturase system isolated from hen liver.

The conversion of stearic acid to oleic acid was 56.9% in the absence of cyclopropene inhibitors and 2.9%, 3.5% and 5.2% in the presence of 0.005 mM sterculic acid, malvalic acid and sterculyl alcohol respectively, in the hen liver desaturase system. In *Chlorella vulgaris*, James et al. (7), have shown that 2-hydroxy sterculyl alcohol inhibits the desaturation of stearic acid. Nordby et al. (15) had shown earlier that sterculyl alcohol, sterculene and the methyl ether of sterculyl alcohol, when fed to hens, induced pink white discoloration of their eggs. This disorder, following the feeding of cyclopropenes, is invariably associated with high levels of stearic acid in the yolks and it has been suggested that the pink white discoloration may result from inhibition of desaturation of stearic acid in the hen (2). The cyclopropene ring is thus the essential requirement for inhibition of desaturation, and the differences observed in the present study between the activities of the three cyclopropenes must be related to the ability of the inhibitor molecule to locate the cyclopropene ring at the active site of the enzyme.

In rats (5), chicks (4) and plant preparations (7), the synthesis of oleic acid from acetate is not inhibited by sterculic acid to the same extent as is the desaturation of stearic acid. Reiser and Raju (5,6) and Donaldson (3,4) believe that sterculic acid acts at the site of desaturation and have postulated that there must be an alternative pathway of synthesis of oleic acid from acetate which does not involve the desaturation of stearic acid. Evidence for an alternative pathway of oleate synthesis had been obtained earlier by other workers (16,17). In plant tissues James et al. (7) suggest that the locus of action of sterculic acid is prior to the desaturase step, possibly at an acyl transfer step, which is not on the direct pathway of acetate to oleate.

A proposal for the mechanism of inhibition of the desaturase system by sterculic acid can be made on the basis of the following observations: the inhibition is irreversible (2), the desaturase system probably contains a thiol enzyme which is essential for activity (6,18), cyclopropene fatty acids react readily with thiol groups (*10*), sterculic acid with the double bond of the cyclopropene ring at the 9,10 position is a more effective inhibitor than malvalic acid with the double bond at the 8,9 position, and sterculic acid is a more effective inhibitor of 9,10 desaturation than of 10,11 or 11,12 desaturation. It is suggested that sterculic acid or its CoA derivative irreversibly and completely occupies the site normally occupied by a substrate as a result of the formation of a carbon-sulfur bond between the C_9 or C_{10} of sterculic acid and a thiol group in the desaturating site of the enzyme.

ACKNOWLEDGMENTS

This study was supported in part by USDA PL 480 Grant No. Fg-Au-102. G. M. Kellerman and R. A. Williams donated labeled fatty acids and R. Hood gave technical assistance.

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[Received July 1, 1968]

The Enzymic Synthesis of Ganglioside: I. Brain Uridine Diphosphate D-Galactose: N-acetyl-galactosaminyl-galactosyl-glucosyl-ceramide Galactosyl Transferase[1]

MORRIS C. M. YIP[2,3] and **JOEL A. DAIN**, Department of Biochemistry, University of Rhode Island, Kingston, Rhode Island 02881

ABSTRACT

An enzyme which catalyzes the transfer of galactose from UDP-galactose to galNAc-gal-glc-ceramide is described. The enzyme is found mainly in the nervous tissue of tadpole (Taylor and Kollros stage 17), adult frog, adult and 8 day old rat. The enzymic activity is localized in the 11,500 x *g*, 20,000 x *g* and 100,000 x *g* particles. The UDP-galactose: galNAc-gal-glc-ceramide galactosyl transferase can be solubilized from the particles by treatment with sodium desoxylcholate and Triton X-100. The pH optimum for the solubilized enzyme is between 6.8 and 7.0 in cacodylate buffer, and the Km is 4.25 x 10^{-5} M. The enzymic reaction is proportional to time for 4 hr and to the amount of protein added. The product of the transferase reaction, using galNAc-gal-glc-ceramide as lipid acceptor, is gal-galNAc-gal-glc-ceramide. A pathway for the biosynthesis of brain gangliosides requiring UDP-galactose: galNAc-gal-glc-ceramide galactosyl transferase is proposed.

INTRODUCTION

Gangliosides are a family of carbohydrate-containing sphingolipids which are found primarily in vertebrate brain (1). The major components of this group of sphingolipids have recently been reviewed by Svennerholm (2). The structural studies of Kuhn and Wiegandt (3) have shown one of the four major gangliosides, mononeuraminyl ganglioside, to be:

galactosyl (1→3)-(N-acetyl) galactosaminyl-(1→4)-[(N-acetyl) neuraminyl-(2→3)]-galactosyl (1→4)-glucosyl ceramide

Basu et al. (4,5) and Kaufman et al. (6) have described four glycolipid transferases from a particulate fraction of embryonic chick brain which can carry on the stepwise synthesis from gal-glc-cer to neuNAc-gal-galNAc-(neuNAc) gal-glc-cer, as follows:

gal-glc-cer —(CMP - neuNAc; N-acetylneuraminyl transferase)→ neuNAcgal-glc-cer

neuNAcgal-glc-cer —(UDP - galNAc; N-acetylgalactosaminyl transferase)→ galNAc-(neuNAc)gal-glc-cer

galNAc-(neuNAc)gal-glc-cer —(UDP - gal; Galactosyl transferase)→ gal-galNAc-(neuNAc)gal-glc-cer

gal-galNAc-(neuNAc)gal-glc-cer —(CMP - neuNAc; N-acetylneuraminyl transferase)→ neuNAcgal-galNAc-(neuNAc)gal-glc-cer

However, Kaufman et al. (6) and Arce et al. (7) have demonstrated an active CMP-neuNAc: gal-galNAc-gal-glc-cer N-acetylneuraminyl-transferase in embryonic chicken brain and young rat brain, respectively. The product of the above transferase reaction in embryonic chicken brain is mononeuraminyl ganglioside and dineuraminyl ganglioside. Because the biosynthesis of asialoganglioside is not known, its importance as a possible precursor for ganglioside formation has not been postulated.

This paper reports the enzymic synthesis of gal-galNAc-gal-glc-cer from galNAc-gal-glc-cer and UDP-galactose.

EXPERIMENTAL PROCEDURE

Preparation of Glycolipids

The method of Ledeen and Salsman (8) was used for the preparation of galNAc-gal-glc-cer, gal-glc-cer and glc-cer from galNAc (neuNAc)gal-glc-cer Fifty milligrams of galNAc-(neuNAc)gal-glc-cer, isolated from brain tissue of an individual with Tay-Sachs disease by the method of Folch et al. (9,10), were hydrolyzed in 16 ml of 0.1 N HCl at 100 C for 15 min. Five volumes of chloroform-methanol

[1] A preliminary report was presented at the 156th National Meeting of the American Chemical Society, Atlantic City, 1968.

[2] Previous publications under the name of Morris G. S. Mark.

[3] Present address: Department of Biological Chemistry, Harvard Medical School and the Cancer Research Institute, New England Deaconess Hospital, Boston, Mass. 02215.

TABLE I

Incorporation of Radioactivity from ^{14}C-UDP-galactose Into Organic Soluble Product in the Presence of N-acetylgalactosaminyl-galactosyl-glucosyl-ceramide in Various Tissues

Tissues	^{14}C-Galactose incorporated (cpm/mg protein)	
	(-) Acceptor	(+) Acceptor
Tadpole brain	206	9160
Adult frog		
brain	320	5550
kidney	174	580
liver	94	226
Adult rat		
brain	923	1915
kidney	190	430
liver	344	990
Eight day old rat		
brain	507	1860
kidney	620	890
liver	560	1240
small intestine	58	252

TABLE II

Subcellular Distribution of the UDP-galactose: N-acetylgalactosaminyl-galactosyl-glucosyl-ceramide Galactosyl Transferase in Adult Frog Brain

Fraction	^{14}C-Galactose incorporated (cpm)	cpm/mg Protein
Whole homogenate	1483	872
Whole homogenate, boiled control	50	29
900 x *g* resuspended particle	90	225
11,500 x *g* resuspended particle	376	671
20,000 x *g* resuspended particle	570	3563
100,000 x *g* resuspended particle	776	5173
100,000 x *g* supernatant solution	89	296

(2:1 v/v) were added, and the solution was separated into two phases. Two additional washes with theoretical upper phase containing water (11) were performed, and the lower organic phase was evaporated to dryness. The material was dissolved in chloroform-methanol (2:1 v/v) and chromatographed on thin layer Silica Gel G plates for a 1 hr ascending run at 22 C with chloroform-methanol-water (61:32:7 v/v/v) (12,13) as the developing solvent. The migration of the compounds was detected with iodine vapors. Under the above conditions, the R_f values for glc-cer, gal-glc-cer, galNAc gal-glc-cer and unhydrolyzed galNAc (neuNAc)gal-glc-cer were 0.8, 0.61, 0.41 and 0.2, respectively. The individual components were scraped off the plates and eluted from the Silica Gel G with chloroform-methanol-water (61:32:7 v/v/v).

The gal-galNAc-gal-glc-cer was prepared by acid hydrolysis (0.1 N HCl) of bovine brain gangliosides for 1 hr at 100 C. The material was dialyzed and the dialysand lyophilized. The gal-galNAc-gal-glc-cer was prepared by preparative thin layer chromatography as described. The developing solvent was chloroform-methanol-water (70:30:4 v/v/v). Under these conditions, the unhydrolyzed ganglioside remained at the origin and the gal-galNAc-gal-glc-cer migrated 1.1 cm from the origin. The calculated R_f value for gal-galNAc-gal-glc-cer was 0.09.

The homogeneity of the purified galNAc-gal-glc-cer and gal-galNAc gal-glc-cer was ascertained by thin layer chromatography in six solvent systems: (1) chloroform-methanol-water (70:30:4 v/v/v) (2) chloroform-methanol-water (61:32:7 v/v/v), (3) chloroform-methanol-conc. ammonia (70:30:5 v/v/v), (4) chloroform-methanol-water- conc. ammonia (60:35:6:2 v/v/v/v) (5) *n*-propanol-conc. ammonia-water (70:15:15 v/v/v). Each glycolipid, when visualized by the orcinol reagent (14), migrated as a single spot in the above six solvent systems. The mixture of glycolipids used as carrier for routine enzymic assay was prepared by acid hydrolysis of ganglioside as described. The dialysand was lyophilized and used.

MATERIALS AND METHODS

Purified galNAc-(neuNAc)gal-glc-cer, gal-glc-cer and gal-galNAc-gal-glc-cer, gifts of J. Kanfer, National Institutes of Health, Bethesda; glc-cer, gift of A. Rosenberg, Columbia University, New York; radioactive CMP-neuNAc, gift of J. Hickman, National Institutes of Health, Bethesda. Uniformly labeled ^{14}C-UDP-galactose, New England Nuclear Corporation and International Chemical and Nuclear Corporation; unlabeled UDP-galactose, Calbiochem; Tween 80, Sigma Chemical Co.; Triton X-100 and Triton CF 54, Rohm and Haas; Sodium desoxycholate, Mann Research Laboratories; Sodium cholate, General Biochem. Inc.; PPO and POPOP, New England Nuclear Corporation and Nuclear Chicago Corporation; thin layer plates of Silica Gel G (250 μ), Analtech Incorporated; mature *Rana pipiens*, J. M. Hazen and Co.; rats, Charles River Co.

The methods described by DeRobertis et al. (15-17) were used with slight modifications. Adult frog brains were homogenized in 0.25 M sucrose solution containing 0.11%

TABLE III

Solubilization of UDP-Galactose: N-acetylgalactosaminyl-galactosyl-glucosyl-ceramide Galactosyl Transferase in Adult Frog Brain Under Different Experimental Conditions

Experimental condition	cpm/mg Protein	Total radioactivity incorporated (cpm)
Control fraction	4500	121,500
11,500 - 100,000 x *g* resuspended particle in sucrose medium	280	588
11,500 - 100,000 x *g* resuspended particle in sucrose medium, frozen and thawed (twice).	1113	5,800
11,500 - 100,000 x *g* resuspended particle in 0.5% sodium cholate	4182	16,600
11,500 - 100,000 x *g* resuspended particle in 0.5% sodium desoxycholate	17,700	310,000
11,500 - 100,000 x *g* resuspended particle in 0.5% in Triton X-100	18,046	228,000
Sonication of the 11,500 - 100,000 x *g* resuspended particle in sucrose medium for 10 min.	207	1,370

2-mercaptoethanol in a ratio of 2 brains per milliliter. A glass homogenizer with a loose Teflon pestle was used. The homogenization was divided into two 1 min periods with an interval for cooling. The total homogenate, diluted with an equal volume of the solution, was submitted to a series of centrifugations. All steps mentioned above were performed at 0 C.

The morphological examination of various particulate fractions was carried out with a RCA EMU-3G electron microscope, after fixation of the pellets in osmium tetroxide, embedding in Epon 812 and thin sectioning.

Preparation of Reaction Mixture

Chloroform-methanol solutions of substrates and detergents were mixed and taken to dryness. Cacodylate buffer (0.04 ml), Mn^{++} (0.02 ml), Mg^{++} (0.02 ml), UDP-galactose-^{14}C (0.02 ml), enzyme and water were added to a final volume of 0.2 ml. The above mixtures were agitated with a Vortex mixer and the tubes placed in a water bath at 37 C.

Enzyme Assay

The enzymic product was determined by measuring the amount of organic-soluble radioactivity produced. After incubation of the reaction mixture, the enzymic reaction was stopped by the addition of 20 vol of chloroform-methanol (2:1 v/v). A mixture of glycolipids (200 μg) was added as carrier, and the solution was partitioned with 0.2 vol of 0.1 N KCl. The upper phase was removed and the lower phase washed 4 times with equal volumes of theoretical upper phase containing 0.1 N KCl (11). The lower phase was transferred to a counting vial and dried under vacuum. To the dried vial, 1 ml of hot toluene was added followed by 10 ml of scintillation fluid (4 g of PPO and 50 mg of POPOP in 1 liter of toluene) and counted by a

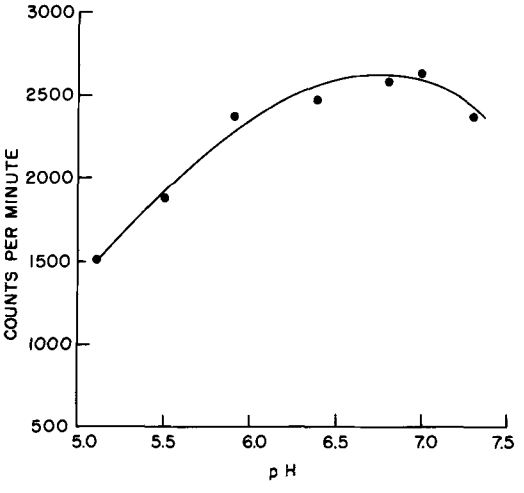

FIG. 1. Effect of pH on the UDP-galactose: galNAc-gal-glc-cer galactosyl transferase activity. The incubation time was 4 hr at 37 C. Protein (0.126 mg) was determined by Lowry's method (18).

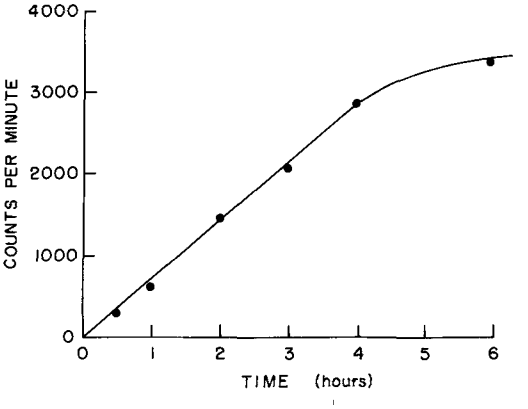

FIG. 2. Time course of the UDP-galactose: galNAc-gal-glc-cer galactosyl transferase reaction. The incubation time was varied at 37 C. Protein (0.126 mg) was determined by Lowry's method (18).

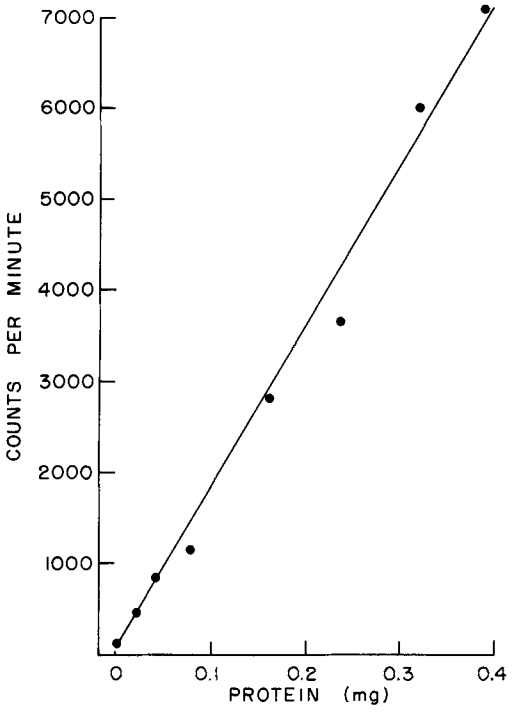

FIG. 3. Proportionality between the amount of protein added and product formed. The incubation time was 4 hr at 37 C. Protein was determined by Lowry's method (18).

liquid scintillation spectrometer. The protein content was determined according to Lowry et al. (18) or the biuret method (19).

RESULTS

Distribution of the UDP-galactose: N-acetylgalactosaminyl-galactosyl-glucosyl-ceramide Galactosyl Transferase Activity in Various Tissues

The distribution of the transferase activity in various tissues of mature frog, adult rat, 8 day-old rat and in tadpole brain (Taylor and Kollros (20) stage 17) is shown in Table I. The tissues were homogenized in a solution containing 0.25 M sucrose and 0.11% 2-mercaptoethanol. The suspensions were centrifuged at 11,500 x *g* for 20 min and 50 μliters aliquots of the supernatant were assayed for the transferase activity. In addition to the tissue extracts, the incubation mixtures contained 4 μmoles of Mn^{++}, 4 μmoles of Mg^{++}, 20 μmoles of cacodylate buffer (pH 7.0), 83.6 mμmole N-acetyl-galactosaminyl-galactosyl-glucosyl-ceramide, 140 mμmole ^{14}C UDP-galactose (3.7×10^5 cpm/μmole), 0.4 mg of Triton CF-54, 0.2 mg Tween 80 and water in a volume of 0.2 ml. The incubation time was 4 hr at 37 C. Under the

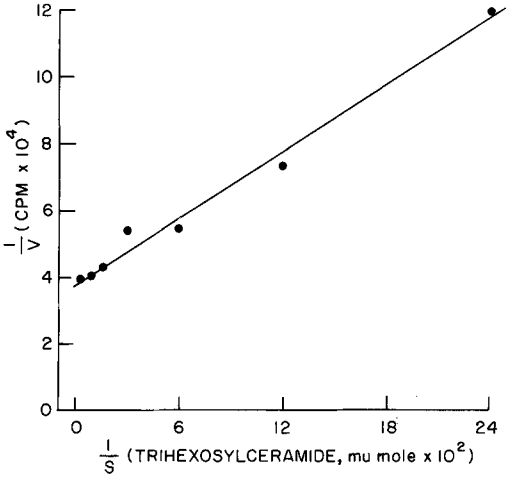

FIG. 4. Lineweaver-Burk plot of the reciprocal of the velocity of UDP-galactose: galNAc-gal-glc-cer galactosyl transferase vs. the reciprocal of the galNAc-gal-glc-cer concentration. The incubation time was 4 hr at 37 C. Protein (0.156 mg) was determined by Lowry's method (18).

above conditions, the reaction was proportional to the amount of protein added and the time of the reaction. Protein was determined by biuret method. Tadpole (Taylor and Kollros stage 17) was used.

The highest activities were observed in the homogenates of the tadpole brain and adult frog brain. Partial purification of the enzyme was undertaken in the adult frog brain.

Partial Purification of the UDP-galactose: N-acetyl-galactosaminyl-galactosyl-glucosyl-ceramide Galactosyl-Transferase

Assay of fractions obtained by differential centrifugation revealed that most of the enzyme activity was contained in the microsomal fraction which sedimented between

TABLE IV

Requirements for the UDP-Galactose: N-acetylgalactosaminyl-galactosyl-glucosyl-ceramide Galactosyl Transferase

System	^{14}C-Galactose incorporated (cpm)
Complete system	3160
Minus galNAc-gal-glu-cer	570
Minus MN++	510
Minus Mg++	3190
Minus MN++ and MG++	489
Minus detergent mixture (Triton CF-54 and Tween 80)	2995
Plus 0.28 μmole galactose	3029
Plus 0.28 μmole UDP-Galactose	856
Plus 6 μmoles of EDTA	103

TABLE V

The R_f Values of the Radioactive Product and the Standard Glycolipid in Various Solvent Systems

Solvent system	R_f	
	Radioactive product	Standard gal-galNAc-gal-glu-cer
Chloroform-methanol-water[a] (70:30:4 v/v/v)	0.09	0.09
Chloroform-methanol-water[a] (61:32:7 v/v/v)	0.34	0.33
Chloroform-methanol-conc. ammonia[a] 70:30:5 v/v/v)	0	0
Chloroform-methanol-water-conc. ammonia[a] 60:35:6:2 v/v/v/v)	0.20	0.20
n-Propanol-conc. ammonia-water[b] (70:15:15 v/v/v)	0.23	0.25
n-Propanol-conc. ammonia-water[b] (60:20:10 v/v/v)	0.34	0.32

[a]Ascending thin layer chromatography for 1 hr at 22 C.
[b]Ascending thin layer chromatography for 2 hr at 22 C.

11,500 and 100,000 x *g* (Table II). Forty-one adult frog brains were homogenized in sucrose solution as described in the Experimental Procedure section. The homogenate was centrifuged for 10 min at 900 x *g* at 0 C. The precipitate was washed twice in sucrose medium and centrifuged to obtain the 900 x *g* particle. The supernatant fluid was pooled and centrifuged at 11,500 x *g* for 20 min at 0 C. The precipitate was washed once in the same medium and centrifuged again to obtain the 11,500 x *g* particle. The supernatant fraction was combined and centrifuged at 20,000 x *g* for 30 min at 0 C. The precipitate was designated as the 20,000 x *g* particle and the supernatant fluid was centrifuged at 100,000 x *g* for 1 hr at 0 C. The precipitate was denoted as the 100,000 x *g* particle. To obtain the 100,000 x *g* supernatant solution, a small portion of the original homogenate was centrifuged at 100,000 x *g* for 1 hr and the supernatant was used. Each particulate fraction was resuspended in an amount of 0.25 M sucrose containing 0.11% 2-mercaptoethanol equivalent to the volume of the original homogenate. Aliquots of 50 μliter of each fraction was assayed according to the conditions in Table I. Protein was determined by biuret method.

Morphological examination of the 20,000 x *g* and 100,000 x *g* fractions by electron microscopy showed that these fractions are similar to the light and heavy microsomal fractions of rat brain (21). The 11,500 x *g* fractions, which have demonstrable transferase activity, contained free mitochondria and synaptosomes. The synaptosomes showing synaptic vesicles, mitochondria and larger vesicles are similar to fraction B_1 from Bullfrog brain (22) and the mitochondrial fraction from rat brain (23).

The UDP-galactose:galNAc-gal-glc-cer galactosyl transferase could be released from the particles which sedimented between 11,500 and 100,000 x *g* by treatment with Triton X 100 or with sodium desoxycholate (Table III). The fractions were prepared according to the conditions in Table II, except that the 11,500 x *g* supernatant fluid was immediately centrifuged at 100,000 x *g* for 1 hr at 0 C. The precipitate was resuspended in different mediums equal to the volume of the original 11,500 x *g* supernatant, and recentrifuged at 100,000 x *g* for 1 hr at 0 C. The 100,000 x *g* supernatant was used as the source of enzyme, and assayed according to the conditions in Table I. The control fraction contained just the 11,500 - 100,000 x *g* resuspended particles in sucrose medium as the source of enzyme. All detergents were made in sucrose medium (w/v) 0 25 M containing 0.11% 2 mercaptoethanol. Sonication was performed at 10 kc in a Raytheon sonic oscillator and recentrifuged at 100,000 x *g* for 1 hr at 0 C. The supernatant was used for enzyme assay as described. Protein was determined by Lowry's method.

Although Triton X-100 and sodium desoxycholate solubilized only 47% and 65% of the protein respectively, the total radioactivity incorporated in the soluble fractions (228,000 cpm and 310,000 cpm) was 2 to 3 times that of the control (121,500 cpm). These results suggest that both Triton X-100 and sodium desoxycholate stimulate the solubilized

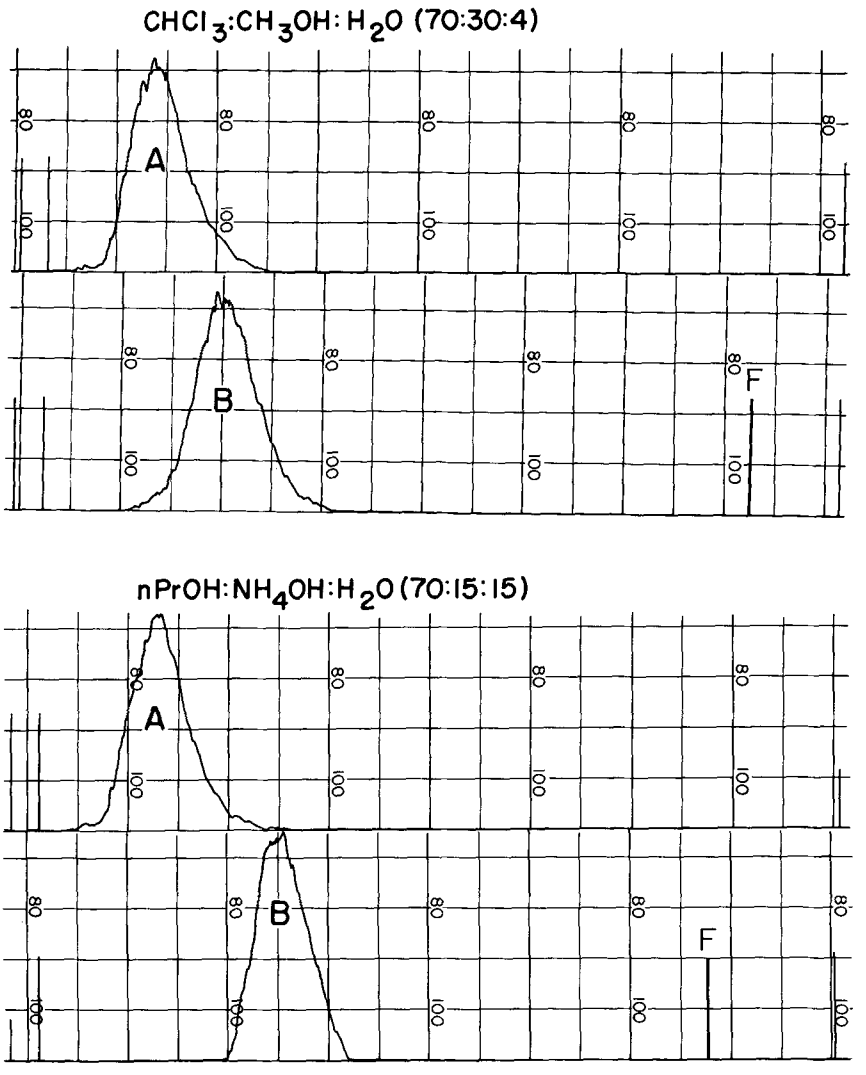

FIG. 5. Reproduction of the radioactive scans of thin layer chromatograms of the enzymic product, gal-galNAc-gal-glc-cer on Silica Gel G plates. A, radioactivity at the origin before thin layer chromatography; B, radioactivity after thin layer chromatography; F, solvent front.

enzyme activity. Conditions such as freezing and thawing, sonication and sodium cholate treatment had little effect on releasing or stimulating the enzyme from the particles.

The enzyme preparation solubilized with sodium desoxycholate, which was used for the subsequent experiments, represents a 20-fold increase in specific activity over the whole homogenate.

Properties of the UDP-galactose: N-acetylgalactosaminyl-galactosyl-glucosyl-ceramide Galactosyl-Transferase

The solubilized enzyme (Table IV) requires Mn^{++} for activity which cannot be replaced by Mg^{++}. The enzyme preparation was solubilize with sodium desoxycholate according to the conditions in Table III. Fifty-microliter aliquots of the soluble fraction were assayed for the enzymic activity (Complete system in Table I).

The demonstration that there is no significant decrease of radioactivity incorporated in the presence of a twofold excess of nonradioactive galactose indicates that UDP-galactose is involved in the reaction. Thus, the incorporation of radioactivity is inhibited by the addition of unlabeled UDP-galactose. Sodium desoxycholate, which was used in solubilizing the enzyme, can replace the detergent mixture of Triton CF-54 and Tween 80.

The optimal pH is between 6.8 - 7.0 (Fig. 1) in cacodylate buffer. The transferase reaction is directly proportional to the reaction time for 4 hr (Fig. 2) and to the amount of protein added (Fig. 3). The half-maximal velocity is obtained at 4.25×10^{-5} M (Fig. 4).

Identification of the Organic Reagent-Soluble Product of the Enzymic Reaction

The standard incubation mixture indicated in Table I was used, except that the enzyme (0.31 to 0.39 mg) was the solubilized preparation and the incubation time was 8 hr. The reaction was stopped by the addition of 4 ml chloroform-methanol (2:1 v/v) and 40 μg of standard gal-galNAc gal-glc-cer, instead of 200 μg of a mixture of glycolipids, were added as carrier. The reaction mixture was then processed as described in the Experimental Procedure Section. The organic reagent-soluble product was applied to thin layer Silica Gel G plates. The position of the radioactive peak and the amount of radioactivity were recorded with a Nuclear-Chicago radiochromatogram scanner, Actigraph III, Model 1006 and a digital integrator, Model 8735 before and after thin layer chromatography in six different solvent systems. All the radioactivity at the origin migrated as a single peak in each solvent system The control experiment without galNAc gal-glc-cer was performed similarly and no radioactive peak was detected. Figure 5 shows the results obtained in two of the solvents used. The R_f value of the radioactive product for each solvent system was calculated as follows: R_f = the distance between peak A and B/ the distance of the solvent front from peak A. The R_f values obtained for the enzymic product corresponded to those of authentic gal-galNAc gal-glc-cer (Table V). Furthermore, the radioactivity could not be separated from gal-galNAc-gal-glc-cer on rechromatography.

To demonstrate that the radioactivity incorporated into gal-galNAc-gal-glc-cer was due to ^{14}C-galactose from ^{14}C-UDP-galactose, the radioactive product was recovered from the Silica Gel G plates and hydrolyzed in 2 N HCl for 6 hr at 100 C. The hydrolysate was filtered through glass wool. The filtrate was taken to dryness and dissolved in a small volume of water. A portion of the radioactive material (4010 cpm) was applied to a descending paper chromatogram on Whatman No. 1 filter paper with *n*-butanol-pyridine-water (6:4:3 v/v/v) as the developing solvent for 39 hr at 22 C. Standard hexoses were detected by a modification of the alkaline silver nitrate dipping technique (24). Under the above conditions, the relative migration values (R_g) of galactose, galactosamine and glucosamine were 0.9, 0.5 and 0.6, respectively. The highest radioactivity (3644 cpm) was confined to the galactose region.

To the remainder of the radioactive material, 100 mg of nonradioactive galactose were added. The mixture was oxidized with 25% nitric acid to yield mucic acid (25) Constant specific activity was obtained after repeated recrystallizations (149 cpm/mg, 140 cpm/mg) which correlated with the theoretical value of 142 cpm/mg. The melting point of the product was 217 C, previously reported 211-212 C and 225 C (24).

DISCUSSION

Our experiments show the presence of an enzyme in brain tissue which catalyzes the synthesis of gal galNAc-gal-glc-cer from galNAc gal-glc-cer and UDP-galactose. The enzyme UDP galactose: galNAc gal-glc-cer galactosyl transferase, appears to be initially bound to particles and can be released in a soluble form in the presence of sodium desoxycholate and Triton X-100. The enzyme seems to be specific for N-acetyl galactosamine at the terminal of the trihexosylceramide, since the corresponding trihexosyl ceramide (gal-gal-glucer) isolated from kidney tissue did not serve as lipid acceptor (26). The cellular distribution of the enzyme correlates with that of the gangliosides in frog and rat brain tissue (26,27), suggesting a possible role of the transferase in the biosynthesis of gangliosides.

Many investigators are concerned with the elucidation of the pathway of ganglioside biosynthesis. Hauser (28) has recently demonstrated an enzymic synthesis of gal-glc-cer from glc-cer and UDP-galactose in rat spleen. In addition, rat brain, kidney and liver have detectable enzymic activity. Handa and Burton (29) have reported an enzymic synthesis of galNAc-gal-glc-cer from gal-glc-cer and UDP-N-acetyl galactosamine in young rat brain. The presence of the above two enzymes and the UDP-galactose:galNAc-gal-glc-cer galactosyl transferase together with the CMP-neuNAc:gal-galNAc-gal-glc-cer N-acetyl neuraminyl transferase (6,7) enables us to postulate the following stepwise synthesis of ganglioside:

glc-cer + UDP-gal ⟶ gal-glc-cer

gal-glc-cer + UDP-galNAc ⟶ galNAc- gal-glc-cer

galNAc-gal-glc-cer + UDP-gal ⟶ gal-galNAc-gal-glc-cer

gal-galNAc-gal-glc-cer + CMP-neuNAc ⟶ Mononeuraminyl ganglioside, Dineuraminyl ganglioside and Polyneuraminyl ganglioside

The observation that UDP-gal: galNAc-(neuNAc)gal-glc-cer galactosyl transferase activity, an enzyme on the other proposed scheme (6-8) for ganglioside biosynthesis, was present in the adult frog brain but could not be detected in the adult rat brain (30) emphasizes the importance of gal-galNAc-gal-glc-cer and galNAc-gal-glc-cer as precursors for the biosynthesis of gangliosides in adult mammalian brain.

ACKNOWLEDGMENTS

J. A. Canick and T. W. Cooper prepared the electron micrographs; Mrs. Gail Yip assisted in the preparation of this manuscript. Henry Drexler and Richard Sanderson gave periodical assistance. This work was supported in part by U. S. Public Health Service research grant (NB-05104) from the National Institute of Neurological Diseases and Blindness, and is partial fulfillment of Ph.D. thesis, by M. C. M. Yip.

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[Received July 29, 1968]

Quantitative Analysis of Free and Bound Fatty Aldehydes: Optimum Conditions for p-Nitrophenylhydrazone Formation

WILLIAM J. FERRELL, JAMES F. RADLOFF and **ANDREW B. JACKIW,**
Department of Chemistry, Biochemistry Division, University of Detroit, Detroit, Michigan 48221

ABSTRACT

A method is described for the analysis of free and bound fatty aldehydes in lipid extracts. By radiolabeling techniques it was shown that the method for measuring free fatty aldehydes when used in the presence of bound fatty aldehydes measures less than 1% of the bound aldehydes. Parameters of time, temperature and optimum acid concentration are reported. A comparison has been made between the present method and other published methods of measuring p-nitrophenylhydrazones with emphasis on recoveries and reactivities. The present method is suitable for measuring as little as 0.02 μmoles of fatty aldehyde. The method has been applied to the analysis of free and bound aldehydes present in various mouse tissues.

INTRODUCTION

The use of p-nitrophenylhydrazone for the quantitative determination of plasmalogens is well established (1-3). In the method of Pries and Böttcher (1) modification was made to allow for the measurement of free fatty aldehydes in the presence of plasmalogens. With the recent establishment of free fatty aldehydes as naturally occurring components of lipid extracts (4-6) it seemed that this method would have much practical application. However, the evaluation of this method by the authors of the work reported here indicated that the conditions specified do not give reproducible results. It was found that in the neutral medium the conversion of standard stearaldehyde to the p-nitrophenylhydrazone derivative was less than 50% complete when compared to the conversion of the same amount of aldehyde under acidic conditions. During the evaluation of the aforementioned method it was noticed that the wash procedure using 50% ethanol caused losses of up to 15% of the hydrazones formed. The above points were established by both spectroscopic and thin layer chromatographic techniques (TLC).

After various modifications, including changes in temperature, acid concentration and the concentration of ethanol in the wash step, the procedure reported here was developed. This method can be used to measure as little as 0.02 μmole of fatty aldehyde.

MATERIALS AND METHODS

Reagents

Pure commercial p-nitrophenylhydrazine was recrystallized twice from methanol. Fresh solutions were prepared daily or high blanks would result.

Pure fatty aldehydes were obtained by converting the alcohol (Eastman Organic Chemicals) to the corresponding aldehyde by oxidation of the tosylate with dimethyl sulfoxide (7,8). The aldehydes were separated from unreacted tosylate by chromatography on silcic acid. Gas liquid chromatography (GLC) (5) showed them to be better than 99% pure.

All solvents were reagent grade and with the exception of methanol and ethanol were redistilled before use.

General Procedure

An aliquot of a total lipid extract is mixed with p-nitrophenylhydrazine and sulfuric acid in methanol. The mixture is heated at 60 C for 25 min and then cooled in an ice bath. Water and hexane are added, the mixture shaken and the layers separated. The hexane phase is washed with 35% ethanol to remove excess-p-nitrophenylhydrazine. An aliquot of the hexane is evaporated almost to dryness, dissolved in 95% ethanol and the absorbance determined at 395 mμ against a reagent blank.

RESULTS

Effect of Time

The time course of the reaction of 0.2 μmole of stearaldehyde with p-nitrophenylhydrazine at 60 C in different acid media is shown in Figure 1. The time course for the reaction using the conditions described by Pries and Böttcher is also presented for comparison.

The data shows that under the present conditions reaction is complete within 25 min and that the absorbance values are higher than those obtained by the method of Pries and Böttcher. Part of the explanation for this is the fact that, using their wash of 50% ethanol, up to 15% of the hydrazone is lost. This figure also shows that their method for free and total hydrazones does not give identical values for equal amounts of standard aldehyde.

TABLE I

Extent of Hydrolysis of Plasmalogens by Various Concentrations of Sulfuric Acid

Sample	dpm	Per cent hydrolysis
Plasmalogen bound aldehydes	162,149	--
Hydrazones (free)[a]	1,493	0.92
Hydrazones (bound)[b]	162,053	99.9
Hydrazones (bound)[c]	161,879	99.8

[a] Hydrazones formed from radiolabeled plasmalogens in the 0.001N sulfuric acid medium.
[b] Hydrazones formed from radiolabeled plasmalogens in the 0.008N sulfuric acid medium.
[c] Hydrazones formed from radiolabeled plasmalogens in the 0.06N sulfuric acid medium.

TABLE II

Recoveries of p-Nitrophenylhydrazones of Various Chain Lengths

Hydrazone shorthand designation	Per cent recovery by method[a] A	B	C	D
2:0	0	0	0	0
4:0	0	0	0	0
8:0	56	53	39	47
10:0	74	72	57	65
12:0	88	88	73	80
14:0	92	91	80	87
16:0	100	99	83	95
18:0	101	100	87	99

[a] A, Present work, 0.008N acid medium. B, Present work, 0.001N or 0.06N acid medium. C, Pries and Bottcher, (1) total medium. D, Wittenberg, et al. (2)

Effect of Acid Concentration

The effect of acid concentration on the reaction of 0.2 μmole stearaldehyde with p-nitrophenylhydrazine is shown in Figure 2.

The sulfuric acid concentration for optimum color formation was determined to be 0.008N. However, it was found that this acid concentration gives complete hydrolysis of plasmalogens and thus is not suitable for analysis of free fatty aldehydes in total lipid extracts. For such an analysis, it seemed that regardless of the reaction conditions, the analysis of standard aldehyde in a medium designed to measure free or total aldehydes should give the same value. Inspection of Figure 2 shows that such a situation exists at acid concentrations of 0.001N and 0.06N. Using these two acid concentrations, identical, super-imposable standard curves were obtained over the range of 0.02 to 0.4 μmole of aldehyde. It remained to be shown that sulfuric acid at a concentration of 0.001N did not hydrolyze plasmalogens and was thus suitable for measuring free aldehydes in total lipid extracts. This was accomplished using radiolabeled plasmalogens prepared biosynthetically. An adult male mouse was injected with 1 μc of acetate -1-^{14}C on each of 7 days. On the last day the mouse was killed 4 hr after injection and the internal organs, consisting of heart, brain, kidneys and liver, were removed and the lipid extracted (9). The phospholipids were separated from other lipid classes by TLC using a solvent system consisting of hexane-chloroform-methanol (73:25:2 v/v/v). In this system the phospholipids remain at the origin while the other lipid classes migrate up the plate (5). The phospholipids were eluted with chloroform-methanol-benzene (1:1:1 v/v/v) and rechromatographed in a system of hexane-isobutanol-methanol (100:3:3 v/v/v) to ensure removal of any labeled free fatty aldehydes. The radioactivity of the bound aldehydes was assessed by acid hydrolyzing (10) and recovering the fatty aldehydes by TLC. The acid released aldehydes were quantified as p-nitrophenylhydrazones (2) and an aliquot was counted in a Packard Tricarb Liquid Scintillation Spectrometer, 314E. A 0.25 μmole aliquot of the radiolabeled plasmalogens, in which the aldehydogenic side chain had a total activity of 162, 149 dpm was carried through the free aldehyde analysis, along with 0.2 μmole of standard stearaldehyde. The hydrazones formed were separated by TLC (hexane-chloroform-methanol, 73:25:2 v/v/v) and eluted with 95% ethanol. An aliquot was quantified at 395 mμ and the remaining

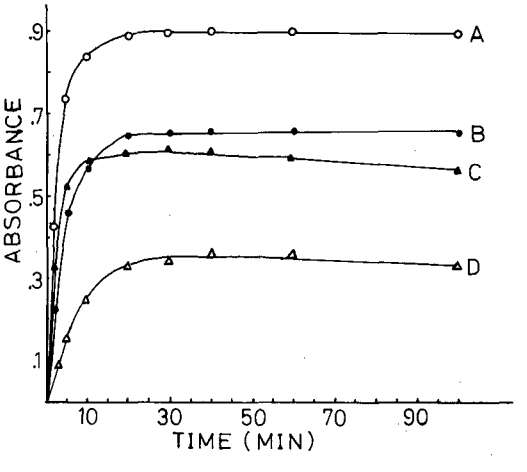

FIG. 1. Time course of p-nitrophenylhydrazone formation from 0.2 μmole stearaldehyde. A and B, present work, 0.008N acid medium and 0.001N or 0.06N acid medium respectively; C and D, Pries and Böttcher (1), total and free aldehyde procedures respectively.

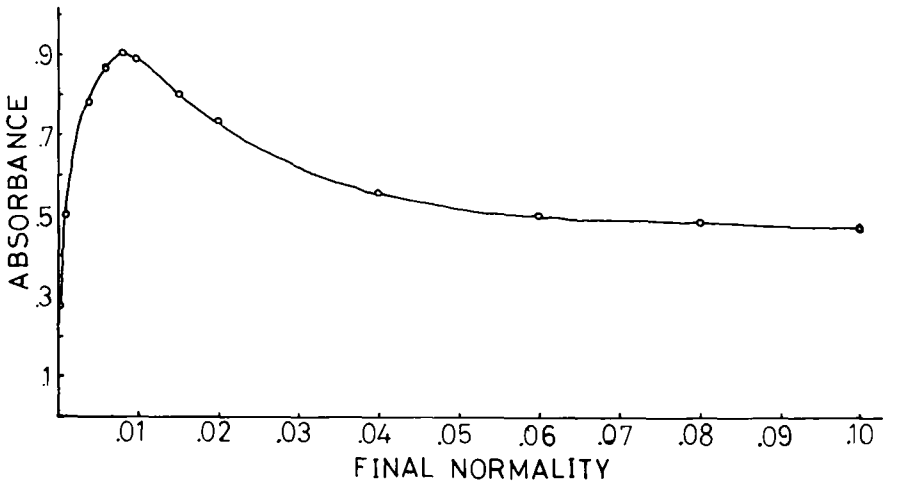

FIG. 2. The effect of sulfuric acid concentration on the reaction between 0.2 μmole stearaldehyde and p-nitrophenylhydrazine. The acid normalities reported are those of the reaction media. Reaction was for 25 min at 60 C.

sample counted in a Packard Tricarb Liquid Scintillation Spectrometer, 314E. The recovery, based on the amount of stearaldehyde added, was 94%. The number of detectable counts in the hydrazones indicated that, at most, there was about 0.9% hydrolysis of the plasmalogens. This data is shown in Table I. The data also shows that complete hydrolysis of the labeled plasmalogens occurs in either the 0.008N or 0.06N acid medium. The importance of this point is twofold: first, the method as described here for free and total hydrazones will measure all of the bound aldehydes present in a lipid extract, and second, in instances where the amount of total aldehydes is low the sensitivity of the method can be increased approximately twofold by running the assay at the optimum acid concentration, since the amount of color formed increases but not the amount of bound aldehydes, when measured relative to a standard curve.

The reason for the formation of color to show acid concentration dependency is not completely understood but a possible explanation is the formation of one or more tautomeric forms of the p-nitrophenylhydrazone which have an increased absorbance at 395 mμ. In any event, the effect of the acid is not on the extraction of the hydrazone into the hexane phase. We have studied the extraction into hexane, of stearaldehyde p-nitrophenylhydrazone from concentrations of sulfuric acid in the range of zero to 2.0N in 72% aq. methanol (v/v) and have observed negligible variations.

Inspection of Figure 2 at acid concentrations of zero and 0.1N once again indicates that the method of Pries and Böttcher for free and total hydrazones does not give equal absorbance readings for a given amount of standard aldehyde.

Effect of Ethanol Concentration in the Wash Step

The effect of ethanol concentration, in the aqueous phase, on the relative solubility of the p-nitrophenylhydrazones in the hexane phase is shown in Figure 3. This curve was determined by measuring the amount of a known aliquot (0.1 μmole) of stearaldehyde p-nitrophenylhydrazone retained by the hexane phase when shaken with aqueous phase containing increasing proportions of ethanol. The results indicate that at 50% ethanol as much as 15% of the hydrazone is removed. This is in agreement with what has been found previously for 2,4-dinitrophenylhydrazones (11). TLC of the 50% ethanol washings using a developing solvent consisting of hexane-chloroform-methanol (73:25:2 v/v/v) gave 2 spots when stained with iodine vapor, one of which had the mobility of p-nitrophenylhydrazine and the other the mobility of the stearaldehyde p-nitrophenylhydrazone. When the infrared (IR) spectrum of the 50% ethanol washings was determined, the presence of p-nitrophenylhydrazone was indicated. If the amount of ethanol was reduced to 35% only the hydrazine spot was observed after TLC and the IR spectrum did not show any hydrazone to be present.

Effects of Aldehyde Chain Lengths

Table II gives a comparison of the recoveries of various chain length p-nitrophenylhydrazones using the present method and two other

TABLE III

Free and Bound Aldehyde Content of Mouse Tissues

Mouse tissue[a]	Bound aldehydes μmoles/100 mg lipid	Free aldehydes μmoles/100 mg lipid
Heart	1.27	.049
Liver	7.23	.098
Kidney	3.48	.052
Brain	8.75	5.38
Skeletal muscle	1.38	0.039

[a]Pooled tissues from 5 male mice with average body weight of 30 g.

published methods (1,2). The recoveries were determined by dissolving known aliquots of p-nitrophenylhydrazone in the appropriate solvent and carrying this aliquot through procedure in question. All values are reported as percentages of the expected absorbance, found by dissolving the same aliquots in 3 ml of 95% ethanol and reading the absorbance at 395 mμ. Identical recoveries were obtained for 0.02, 0.1 and 0.2 μmole aliquots.

Proposed Procedures

Determination of Total Aldehydes (Free and Bound). An aliquot of the lipid sample to be analyzed is placed in a 40 ml conical centrifuge tube and evaporated to dryness. The sample is redissolved in 0.2 ml chloroform. To each sample is added 8 ml 90% aq. methanol (v/v), 1 ml of 0.6N sulfuric acid and 1 ml 0.02 M p-nitrophenylhydrazine in 80% aq. methanol (v/v). The samples are mixed and heated for 25 min at 60 C after which they are cooled in an ice-bath. To each sample is added 10 ml hexane and 5 ml water; the tubes are shaken for 30 sec and the phases separated by centrifugation. A convenient method of discarding the lower phase is to draw it out using a Pasteur pipette attached by rubber tubing to an aspirator. When all but a drop in the point of the conical tube has been removed the rubber tube is pinched off and the pipette withdrawn. Cleaner separations can be achieved by drawing off the lower phase until the upper hexane phase just starts to fill the tip. Since an aliquot of this phase is used the small losses incurred this way are of no consequence. The hexane phase is then washed at least twice with 10 ml 35% aq. ethanol (v/v) by shaking, centrifuging and removing the lower phase as just described. An 8 ml aliquot of the hexane phase is removed and evaporated under nitrogen almost to dryness, the residue dissolved in 3 ml 95% aq. ethanol (v/v) and the absorbance at 395 mμ determined against a reagent blank. It is important that the samples are not allowed to go completely dry as it is extremely difficult to redissolve the hydrazones once they become hard on the sides of the tubes. The amount of total aldehydes present is determined by comparison to an aldehyde standard curve run at the same time, in the manner just described.

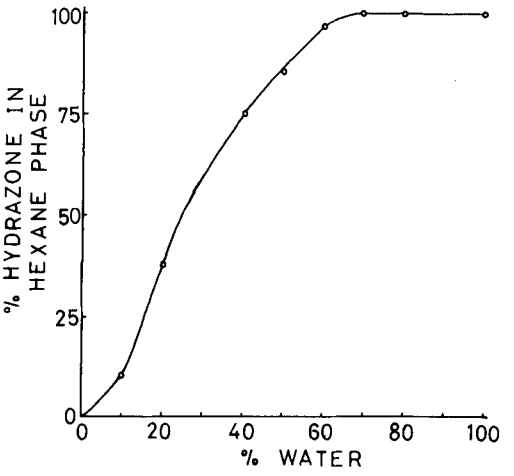

FIG. 3. The effect of composition of the aqueous phase on extraction of 0.1 μmole stearaldehyde p-nitrophenylhydrazone by hexane.

In cases where the amount of total aldehydes is low, increased sensitivity may be obtained by the use of 0.08 N sulfuric acid instead of the concentrations previously mentioned. This is shown in Figure 2, where the final acid concentration for maximum absorbance is seen to be 0.008 N. However, this is not the method of choice since the optimum acid concentration presents a rather sharp peak and therefore slight variations in acid concentration could cause erroneous results.

Determination of Free Aldehydes. For the determination of free aldehydes the general procedure is the same as just outlined with the exception of 1 ml of 0.01 N sulfuric acid is used instead of 0.6 N sulfuric. The standard curve determined under these conditions has a slope equal to that determined using 0.6 N sulfuric, but approximately half that of the standard curve from 0.08N sulfuric. That this method will measure all of the free fatty aldehydes present in a lipid extract was shown in the following way: aliquots of a total lipid extract of mouse hearts containing 139 mg lipid per ml were analyzed for free aldehydes and a value of 0.49 ± 0.02 μmole/100 mg lipid, SD (n=10) was obtained. When the free fatty aldehydes from aliquots of this extract were isolated, as described elsewhere (5), and then analyzed, a value of 0.47 ± 0.03 μmole/100 mg

lipid, SD (n=8) was obtained. The recovery of standard aldehyde using this procedure was 96.7 ± 2.6% SD (n=10).

Quantitation of Free and Total Aldehydes From Total Lipid Extracts

Table III gives the results of analyzing total lipid extracts from various mouse tissues for free and total aldehydes. The lipid extracts were made according to the method of Folch, et al. (9). All samples were stored under nitrogen at -20 C until used.

DISCUSSION

Quantitation of aldehydogenic lipids by p-nitrophenylhydrazone formation involves a number of relationships. The first and most important is the effect of acid concentration. A detailed study of the kinetics and mechanisms of hydrazone formation, as well as other similar reactions involving aldehydes, has been made (12,13). The results of these studies indicate that hydrazone formation is a general acid catalyzed reaction. It has been previously pointed out that there should be an optimum acid concentration (14). Other workers (1) have suggested that identical results can be obtained in either a neutral or acid media, while Wittenberg et al. (2) have stated that without acid no hydrazone was obtained. We have found that while some hydrazone is formed from stearaldehyde in the neutral system, nearly twice as much hydrazone is formed from the same amount of stearaldehyde in the acid medium.

While the method described in this paper has certain similarities to the published methods (1,2) the present method has certain advantages. Decreasing the concentration of ethanol in the wash step eliminates any loss of hydrazone. By careful adjustment of the acid concentration, increased sensitivity can be obtained to measure total hydrazones and, if the free fatty aldehydes are first isolated from lipid extracts (5), this method may be applied to their assessment also. The described procedure, for the analysis of both free and bound aldehydes, has better reproducibility since only one standard curve is used.

The results of the radiolabeled experiments indicate that our method will measure either free or bound fatty aldehydes and, in the case of the latter, essentially 100% of the bound aldehydes present are detected.

ACKNOWLEDGMENTS

This investigation was supported (in part) by research grants from the Michigan Heart Association and the Licensed Beverage Industries, Inc. Arnold Marcantonio gave useful suggestions throughout the course of this work.

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[Received September 25, 1968]

Cis-Trans Isomerization of Unsaturated Fatty Acid Methyl Esters Without Double Bond Migration

D. S. SGOUTAS and **F. A. KUMMEROW**, The Burnsides Research Laboratory, University of Illinois, Urbana, Illinois 61801

ABSTRACT

Trans isomerization of monoenoic and dienoic fatty acid methyl esters has been carried out with thiols and diphenylphosphine in the presence of azobisisobutylnitrile. The equilibrium mixture contained 75-80% *trans* double bonds and there was no migration of the double bonds.

INTRODUCTION

Unsaturated fatty acids can be isomerized from the *cis* to the *trans* configuration by various catalysts, including univalent atoms, molecules with odd electrons, free radicals and paramagnetic substances in general (1,2). Both free radical and ionic mechanisms have been suggested and the formation of addition products and transitional forms as intermediates has been postulated. These intermediates are free to rotate about the axis of the former double bond. After such rotation, dissociation from the catalyst permits reestablishment of the double bond with consequent formation of the geometrical isomer. Side reactions concurrent with the *cis-trans* isomerization include addition (1,3), polymerization (4), hydrogen transfer and shifting of the double bond (5-7). Several studies have demonstrated that reagents isomerizing unsaturated fatty acids differ greatly in their selectivity towards the formation of by-products. Recently, Gunstone and Ismail (7) used thiyl radicals produced by photolysis of diphenyl sulfide and prepared *trans* octadecenoic acids free from positional isomers. In the present paper, monoenoic and dienoic fatty acid methyl esters have been isomerized *cis-trans* with thiyl or phosphinyl radicals produced by radical initiators. The method has been shown to give efficiently and cleanly a definitive *cis-trans* equilibrium and to be totally free of double bond migration. Other advantages that may be cited are: mild reaction conditions with a high yield of product, ready applicability of the reaction to small scale preparation of labeled compounds and simplicity of procedure.

MATERIALS AND METHODS

Methyl palmitate, oleate, petroselenate, linoleate, *cis* vaccenate, elaidate and 11-*cis*, 14-*cis* eicosadienoate were purchased from the Hormel Institute, Austin, Minnesota. Gas liquid chromatography (GLC) indicated that they contained less than 1% impurities. Thin layer chromatography (TLC) on Silica Gel G impregnated with silver nitrate (8), also showed that these esters were pure. Thiophenol, diphenyl sulfide and dodecanethiol were supplied by Aldrich Chemical Co., Milwaukee, Wis., and diphenylphosphine by Orgmet, Inc., Hampstead, N.H. These reagents were fractionally distilled and a center cut used in this study. Solvents were A. R. grade and distilled before use. Azobisisobutylnitrile (AIBN) was used as received (Eastman Chemical Products, Inc. Rochester, N.Y.) 1-^{14}C-oleic and 1-^{14}C-linoleic acids (specific activity 9.0 mc/mmole and 16.1 mc/mmole, respectively) were purchased from Tracerlab, Waltham, Mass. They were purified by chromatography on acid-treated Florisil (9).

The reactions were carried out in glass vials having constricted necks for sealing. In a typical experiment (Exp. 2, Table I), 3 mg of AIBN, 0.2 ml of benzene, 110 mg (1 mmole) of thiophenol, 296 mg (1 mmole) of methyl oleate and 50 mg methyl palmitate, serving as a nonreactive internal standard, were placed in a vial. The reaction mixture was then deaerated by freezing, evacuating and thawing, sealed under vacuum, placed in a constant temperature sand bath preheated to 65 C and maintained at this temperature. Similar conditions were used for other experiments (Table I). At a given time interval, each sample was removed from the bath, cooled, an equivalent amount of silver nitrate in aqueous solution was added to precipitate the thiophenol or diphenylphosphine and the methyl esters were extracted with petroleum ether. They were passed through a silicic acid column and subjected to further analysis. In certain cases the methyl esters were purified by preparative GLC (10% Apiezon L on Celite 545) prior to their analysis.

GLC employing packed columns (15% Apiezon L in chromosorb W 100-200 mesh treated with hexamethyl disilazane at 210 C, or 10% diethylene glycol succinate on chromosorb W 60-80 mesh at 165 C) indicated depletion of catalyst and absence of side reaction products.

The relative amounts of *cis* and *trans* isomers were conveniently measured by capillary GLC using either 200 ft Apiezon L, or diethylene

TABLE I

Cis, Trans-Isomers Equilibria of Unsaturated Fatty Acid Methyl Esters by Thiyl and Phosphinyl Radicals

Exp.	Methyl ester[a]	Reagent[a]	Solvent[a]	Temp. C	Time hr	Isomer distribution % by GLC[b] All *trans*	*cis, trans*	All *cis*	Double bond movement[c]
1	Oleate	Thiophenol	None	65	6	79.		20.8	~0
2	Oleate	Thiophenol	Benzene	65	6	77.6		22.	~0
3	Oleate	Thiophenol	Cyclohexane	65	6	79.8		18.9	~0
4	Oleate	Thiophenol	CCl_4	65	6	81.5		19.2	~0
5	Oleate	Diphenyl-phosphine	Benzene	75	6	79.1		21.3	~0
6	Oleate	Diphenyl-phosphine	CCl_4	75	6	78.1		20.9	~0
7	Oleate	Dodecane-thiol	Benzene	65	6	50.8		49.0	~0
8	Oleate	Diphenyl sulfide	Benzene	65	6	3.6		96.2	~0
9	Petroselenate	Thiophenol	Benzene	65	6	78.5		23.2	~0
10	Vaccenate (*cis*)	Thiophenol	Benzene	65	6	77.6		21.5	~0
11	Elaidate	Thiophenol	Benzene	65	6	76.8		23.5	~0
12	Linoleate	Thiophenol	Benzene	65	8	50.8	40.2	10.5	<2%
13	Linoleate	Thiophenol	Cyclohexane	65	8	50	38.7	12.1	<2%
14	Linoleate	Diphenyl-phosphine	Benzene	75	8	49.5	39.8	11.3	<2%
15	Eicosa-9,12-dienoate	Thiophenol	Benzene	65	8	48	40	12.8	<2%

[a]The reaction mixture contained: 1mmole of reagent per 1 equivalent double bond, 0.2 ml solvent and 3 mg of AIBN.

[b]Expressed as percentage of the total area under the peaks on the chromatogram tracing.

[c]Migration by ozonolysis.

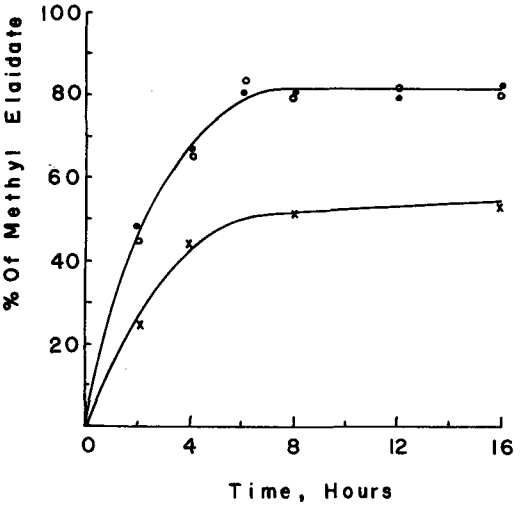

FIG. 1. *Trans* isomerization of methyl oleate with thiophenol (●--●), diphenylphosphine (o--o) and dodecanethiol (x--x). Experiments 2, 5 and 7 (Table I), respectively.

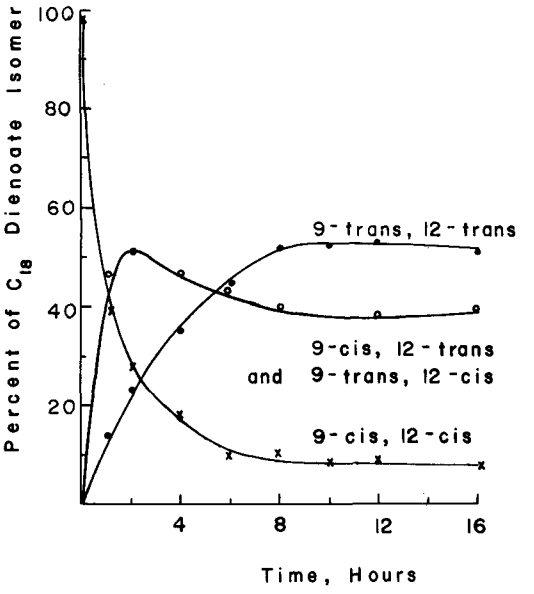

FIG. 2. Isomer composition of randomly *cis-trans* isomerized methyl linoleate using thiophenol catalyst. Experiment 12 in Table I.

glycol succinate capillary columns (10,11).

In addition, GLC provided a quantitative estimate of the isomerized fatty acid methyl esters. Methyl palmitate was used as an internal standard and calibration curves of methyl palmitate and oleate were constructed based on their corresponding peak areas in chromatograms obtained by GLC. From the slope of the calibration curve and the known weight of added methyl palmitate, the amount of *cis* or *trans*, or both, in any given sample could be determined in the reaction products. Alternately, the amount of isolated *trans* bonds was determined in KBr pellets from the absorbance measurements of 675 cm^{-1} (*cis*) and 962 cm^{-1} (*trans*) on an IR spectrometer using a calibration curve.

In order to determine the position of the double bond(s), methyl esters were cleaved to aldehydes and aldehydo-esters by reductive ozonolysis (12). Cleavage products were identified by TLC and quantified by temperature programmed GLC (12). When required, configurational isomers were separated by argentation chromatography either on columns (13) or plates (8).

Radioactive samples were isomerized in the same way and the reaction products analyzed by argentation chromatography (8,13). In a typical experiment conducted in a capillary tube, 1 μmole of methyl 1-^{14}C-linoleate (16.1 μC/μmole) reacted with 2 μmoles thiophenol in 10 μl benzene in the presence of a small crystal of AIBN. The tube was sealed and heated in a sand bath at 65 C for 8 hr. The sample was taken up in hexane and a small aliquot was mixed with 50 mg of methyl linoleate isomerized under similar conditions (Exp. 12, Table I) and the mixture was subjected to column argentation (13) and thin layer argentation (8) chromatography. Radioactivity was monitored in the column effluent and in the Silica Gel scrapings. Counts were made in a scintillation counter.

RESULTS AND DISCUSSION

The results of the rate studies with equivalent amounts of methyl oleate and thiophenol, dodecanethiol or diphenylphosphine are shown in Figure 1. The per cent of isolated *trans* bonds were determined from IR and capillary GLC data. Thiophenol gave a much faster reaction over dodecanethiol, probably due to the composite radical formed from addition of the thiol to the double bond (15,16) which was resonance stabilized in the case of thiophenol. By analogy diphenylphosphine gave similar rates to thiophenol. After 5 hr an oleic:elaidic acid equilibrium was obtained and the final product contained 80-82% *trans* bonds. This value closely agreed with the equilibrium ratio of elaidic:oleic when either selenium or nitrous acid was used as catalyst (17). Using the calibration curve of the internal standard, recoveries of better than 97% of monoenoic acids were obtained pointing to the absence of side reactions. Capillary GLC and double bond cleavage

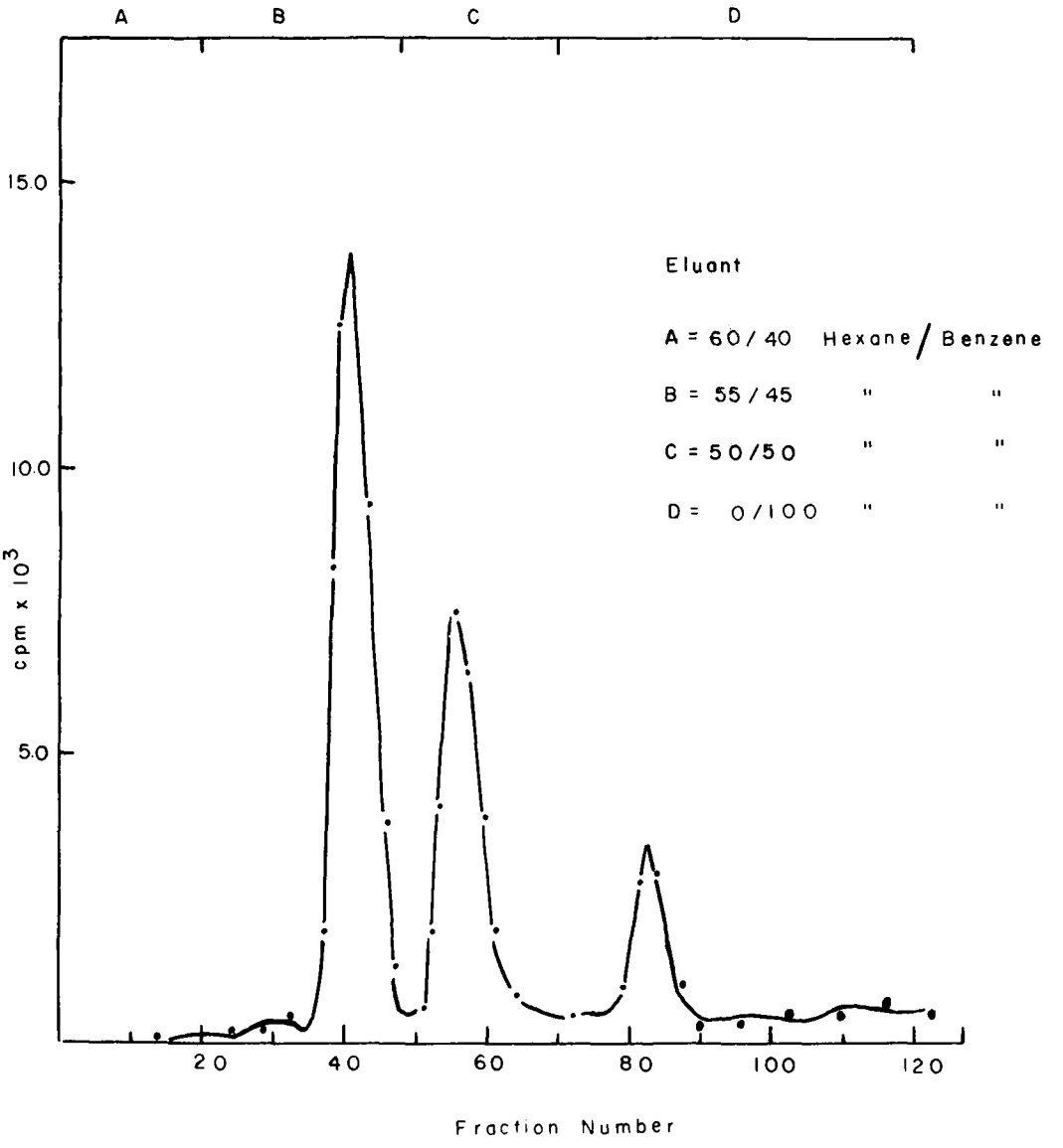

FIG. 3. Liquid-solid chromatography of isomerized methyl 1-^{14}C-linoleate. Eluted in sequence 9-*trans*, 12-*trans*; mixture of 9-*cis*, 12-*trans* and 9-*trans*, 12-*cis*; and 9-*cis*, 12-*cis* methyl octadecadienoate. Prior to chromatography, the radioactive sample was diluted with carrier methyl linoleate isomerized under similar conditions.

analyses indicated absence of detectable amounts of positional isomers.

Data on methyl linoleate isomerized with thiophenol showed (Fig. 2) that about 75% *trans* bonds were present at equilibrium. *Trans* bonds were determined from IR data after the correction suggested by Scholfield et al. (18) was applied. Capillary GLC indicated that the actual isomer content found at equilibrium was approximately 10% 9-*cis*, 12-*cis*; 17% 9-*trans*, 12-*cis*; 18% 9-*cis*, 12-*trans* and 53% 9-*trans*, 12-*trans* methyl octadecadienoate. Ultraviolet, GLC and double bond cleavage analyses indicated absence of conjugated as well as positional isomers. Yields based on the internal standard approximated 94% to 95% for the recovered stereoisomers. However, on longer reaction times or in excess of catalyst, or both, the overall yield decreased and the formation of by-products was noticed (Sgoutas, manuscript in preparation).

Data from various unsaturated methyl esters

isomerized in the presence of thiophenol, diphenylphosphine and dodecanethiol are presented in Table I. The reaction was stopped at time intervals when equilibrium was attained as indicated by the rate studies (Fig. 1 and 2). For the methyl esters tested, the *cis:trans* ratio varied little with the position of the double bond and there was no pronounced solvent effect. Diphenyl sulfide was inert.

Walling and Helmreich (15) and Pellon (19) studied in detail the radical additions of thiols and phosphines to olefins and they have proposed a multistep chain mechanism. Conceivably the same scheme can describe the addition of thiyl and phosphinyl radicals to the double bond of unsaturated fatty acids. It is assumed that an intermediate radical can regenerate either the *trans-* or *cis*-unsaturated fatty acid depending upon its conformation at the time of the thiyl elimination. For methyl 1-^{14}C-linoleate, isomerized as described, the scheme of separation of the isomeric species is given in Figure 3. The radioactivity distribution was approximately 52.0% in the 9-*trans*, 12-*trans*; 41.5% in the 9-*cis*, 12-*trans* and 9-*trans*, 12-*cis*; and 6.5% in the 9-*cis*, 12-*cis* methyl octadecadienoate. The radioactivity distribution practically matched the mass distribution (Exp. 12, Table I). Data from TLC radioassay substantiated the above findings. However, the radiochemical yield was only 88% at specific activities of 16.1 μc/μmole of methyl 1-^{14}C-linoleate. When methyl 1-^{14}C-linoleate of lower specific activity (16.1 μc/mmole) was isomerized in the same way the radiochemical yield was 92.5%; practically the same as with the chemical yield. The reason for this was not investigated.

Theoretically the method should be applicable to fatty acids with any type or combination of types of polyunsaturation, whether isolated, methylene interrupted or conjugated. The only factor that would make its unqualified use unwise is the possibility that the pol unsaturated fatty acids are susceptible to rac cal polymerization.

ACKNOWLEDGMENTS

Technical assistance by Holly Sanders and Don Dimmett; support by research grant HE 1077 USPHS.

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[Received October 22, 1968]

Triglyceride Sub-Classes of Dog Plasma And Organs

MARTIN GOLD, Gerontological Research Institute
Philadelphia Geriatric Center, Philadelphia, Pennsylvania 19141

ABSTRACT

Lipids were extracted from liver, kidney, myocardium, skeletal muscle, arterial and femoral venous plasma. Triglycerides were purified by thin layer chromatography and the double bond subclasses separated by silver nitrate silica gel H thin layer plates. Gas chromatography was used to delineate the fatty acid composition of each sub-class as well as the carbon number of the purified triglyceride from each site. The major double bond sub-class was O11 (O,saturated fatty acid; 1,one double bond; 2,two double bonds). The plasma values were highest in 001 and 002. All sites had quite uniform 112 while plasma had the lowest 012. The percentage of all saturated fatty acids in a band was summed and equated to 100% and the fatty acid percentage recalculated on this basis. The saturated fatty acid distribution in myocardium and skeletal muscle was highest in myristate in the third 001 sub-class and liver and plasma had the highest stearate in the second 001 band. The palmitate was similar in most bands. Every sample, except the subcutaneous adipose tissue, had a maximum triglyceride content of carbon number 52 (the carbon number represented the sum of all carbon atoms in the fatty acids present in a molecule). The liver and subcutaneous adipose tissue triglyceride carbon number 52 and 54 were approximately equal. The liver exhibited the largest 56.

INTRODUCTION

A report of dog adipose tissue triglyceride double bond sub-classes from various sites was recently presented (1). This paper is a continuation of the study to define, in a dog fed a general meat diet ad lib., the triglyceride double bond sub-classes in the various organs. The first report concerned the perirenal, pericardial, mesenteric and subcutaneous adipose tissue, while this report details the results for liver, kidney, myocardium, skeletal muscle, arterial and femoral venous plasma samples.

METHODS

A male mongrel dog weighing 20 kg was fed ad lib. on Thrivo or Ken-L-Ration for at least one month in the animal colony at Hahnemann Medical College. The dog was anesthetized with Nembutal and exsanguinated. Blood samples were taken from the carotid artery and femoral vein. A sample of liver, leg skeletal muscle, whole kidney and myocardium was removed. Adipose tissue was trimmed away from the tissues wherever appropriate. The tissue samples were weighed and immediately honogenized in a Waring Blender with 20 vol of chloroform-methanol (2:1), filtered into a separatory funnel and overlayed with 0.2 vol of physiological saline and allowed to stand overnight. The lower chloroform layer was removed, dried with anhydrous magnesium sulfate and evaporated to a small volume in a Buchler rotary evaporator under vacuum. The liquid was placed in a tube and evaporated to dryness under nitrogen. The residue was dissolved in hexane and the tube sealed with a teflon lined cap and stored at -20 C.

Blood was centrifuged, the plasma removed, 20 vol of chloroform-methanol (2:1) added, and the mixture treated as indicated above.

An antioxidant, 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline, Santoquin, (K and K Laboratories, Inc.) was added to the homogenates and stored material.

A detailed statement of the methodology was given in a previous report (1).

Silica gel G thin layer plates (0.5 mm, 20 x 20 cm) were utilized to purify triglycerides using a solvent system of hexane-ethyl ether-acetic acid (78:20:2). The developed plate was sprayed with 0.02% dichlorofluorescein (pH 7) for visualization and the bands identified by standards (Applied Science, Inc.). Triglycerides were eluted with 5% methanol in ether.

Triglyceride double bond sub-classes were separated on 25% silver nitrate-impregnated silica gel H (wt/wt) thin layer plates (0.5 mm) using a wedge cut into the gel and a two step development. The first solvent was ethyl ether-benzene (10:90) and the second was benzene-petroleum ether (bp 36-52 C), (80:20).

Fatty acid composition was analyzed after methylation with methanol and sulfuric acid and quantitated by using heptadecanoic acid as an internal standard on a diethylene glycol succinate polymer column in a Beckman GC-4 gas chromatograph.

The triglyceride carbon number was determined by gas liquid chromatography. A stain-

TABLE I

Triglyceride Fatty Acid Composition in Various Organs of the Dog (Mole %)

Organ	Fatty acid carbon length; number of double bonds							
	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4
Myocardium	2.9	22.0	6.4	7.8	43.2	16.9	0.8	Trace
Muscle	3.0	24.7	7.9	7.0	43.8	13.5	Trace	Trace
Liver	1.8	21.6	3.7	12.4	44.0	13.0	0.8	3.0
Kidney	2.8	26.5	4.5	7.0	40.1	15.0	0.9	3.4
Arterial plasma	1.9	22.8	3.1	9.8	43.7	15.7	0.7	2.3
Venous plasma	1.6	23.5	3.0	10.4	43.3	16.0	Trace	2.1

less steel column 2 ft x 1/8 in O.D. was silanized with 5% dichlorodimethylsilane in chloroform and packed with 3% SE-30 on Gas Chrom Q (80-100 mesh). The column was thoroughly conditioned by heating at 350 C for several days, the oven temperature was then programmed from 250 C to the temperature required to elute tristearin at a rate of 2.5 C/min. This was continued until the standards (saturated triglycerides from carbon number 42 to 54, Applied Science, Inc.) produced a constant peak area. Temperature programming, starting at 250 C and rising at 4 C/min, was used for all samples, continuing until all peaks were eluted. Response factors were determined and each peak appropriately corrected (2). Helium was the carrier gas. Since higher flows did not produce increased resolution, 40 ml/min of helium was used; the inlet temperature was 325 C and the detector temperature 375 C. A Beckman model GC-4 was preferred as it gave better results than several other available instruments, perhaps because of less dead space and better temperature control. Trimyristin was added to each sample as an internal standard. This was used in preference to other internal standards, e.g., tridecanoin, since less time per run was involved and assays without internal standard indicated that the amount of carbon number 42 or less was smaller than 0.2% of the total area.

Peak areas were determined from the height and width at half height measurements and the mole per cent calculated from experimentally derived response factors.

RESULTS

Table I contains the major fatty acid composition of triglycerides of myocardium, skeletal

TABLE II

Per Cent of Total Triglyceride Based on Unsaturation Classes in Various Organ Sites in Dog (Mole %)

Double bond sub-classes	Myocardium	Skeletal muscle	Liver	Kidney	Arterial plasma	Venous plasma
000	1.9[b] ±0.5	2.6 ±0.3	1.9 ±0.5	2.9 ±0.5	4.3 ±1.1	7.0
001	17.0 ±1.5	17.8 ±0.6	13.9 ±0.4	17.6 ±0.6	20.9 ±2.0	19.3
011	22.7 ±1.3	27.7 ±1.5	22.1 ±2.2	27.5 ±1.3	20.2 ±0.5	15.5
002	5.5 ±0.4	5.9 ±0.1	5.0 ±0.7	4.1 ±0.8	5.7 ±0.9	8.6
111	10.8 ±0.3	12.1 ±0.1	11.8 ±0.7	9.9 ±0.8	8.7 ±3.1	7.1
012	19.7 ±0.8	17.0 ±1.4	17.2 ±1.5	17.7 ±1.0	14.8 ±0.7	15.4
112	9.3 ±1.8	9.5 ±1.0	9.4 ±0.5	10.5 ±0.2	11.7 ±0.0	10.3
022	2.7 ±0.2	2.0 ±0.5	2.8 ±0.2	3.5 ±0.2	3.7 ±0.8	3.2
Others	10.3 ±1.6	5.3 ±0.1	16.0 ±0.3	6.4 ±0.8	10.2 ±0.5	13.8

TABLE III

Saturated Fatty Acid Composition of Double Bond Sub-Classes in Various Dog Plasma and Tissue Sites Based on 100% (Mole %)[a]

	000	001	001	001	011	002	012	022	Average	
Myocardium										
14:0	11	15	5	13	5	10	4	3	8	-
16:0	62	60	64	63	65	67	64	60	63	-
18:0	26	26	32	19	29	23	33	36	28	-
Skeletal muscle										
14:0	11	13	4	14	7	11	5	3	9	-
16:0	68	62	69	74	72	71	74	71	70	-
18:0	20	22	27	12	20	18	22	27	21	-
Liver										
14:0	9	8	4	10	6	3	2	4	6	6[b]
16:0	57	57	51	62	62	63	65	62	60	61
18:0	35	36	45	28	33	33	34	32	34	33
Kidney										
14:0	10	8	5	10	5	8	4	Trace	6	-
16:0	68	71	65	74	71	71	67	69	69	-
18:0	23	22	31	17	25	22	28	31	25	-
Arterial plasma										
14:0	7	11	4	9	5	7	4	3	6	6[b]
16:0	64	70	54	67	66	65	65	66	65	66
18:0	29	19	43	25	30	29	31	31	30	28
Venous plasma (femoral)										
14:0	9	12	6	10	9	Trace	7	Trace	6	7[b]
16:0	63	57	52	65	66	88	65	69	66	68
18:0	29	32	42	25	26	12	28	31	28	26

[a]Myocardium, kidney and liver represent the average of quadruplicates; skeletal muscle triplicates, arterial plasma duplicates and venous plasma represent a single sample.

[b]The average calculated without the second 001 band.

muscle, liver, kidney, and arterial and venous plasma. The values are fairly uniform, although kidney and muscle had somewhat higher palmitic acid while myocardium and muscle had more palmitoleic. Liver, arterial and venous plasma showed more stearic acid and liver and muscle contained less linoleic acid. Kidney exhibited the lowest oleic acid.

The double bond separation by silver nitrate thin layer chromatography of the triglycerides isolated from various tissues is shown in Table II The three 001 sub-classes were summed and expressed as a single entity. The position of the fatty acid on the glycerol moiety was not determined. The arterial and femoral venous plasma differed in the 011 sub-class but were similar in all other respects. When the plasma values were compared to the others, it could be noted that they were highest in 001 and 002. All organs had quite uniform 112 while plasma had the lowest 012. The 011 sub-class was present in the largest quantity.

The samples were analyzed at least as duplicates. However, not enough triglyceride was available for more than one run of the venous sample.

Table III shows the data for saturated fatty acid distribution in the double bond sub-classes. The percentages of saturated fatty acids were summed, equated to 100% and the individual percentages recalculated. Myocardium and skeletal muscle had the highest myristate in the third 001 sub-class while liver and plasma had the highest stearate in the second 001. The palmitate was similar in most bands. Skeletal muscle demonstrated the highest palmitate and the lowest stearate average. Liver exhibited the highest stearate and along with plasma the lowest palmitate overall. Kidney palmitate was as high as that of the skeletal muscle. In general, the second 001 sub-class had the largest amount of stearate. This result was especially pronounced in the liver and the plasma samples.

The triglyceride carbon number distribution in different tissues and plasma was determined and the results are presented in Table IV. The carbon number is the sum of the carbon atoms in the fatty acids in the molecule, e.g.. tristearin has a carbon number of 54. The most striking observation was the regularity of the pattern of increase and then decrease in the carbon number. Every sample, except the subcutaneous adipose tissue, had a maximum content of carbon number 52 which averaged 42.8%.

Three carbon number classes (50, 52 and 54) had over 90% of the fatty acids, as would be expected from the fatty acid carbon chain content of the samples. The liver and subcutaneous adipose triglyceride carbon number 52 and 54 were roughly equivalent, while in the other samples the 52 was larger. The liver exhibited the largest, 56.

DISCUSSION

The fatty acid composition of triglycerides in the various organs of the dog was not strikingly dissimilar. However, it can be noted that the plasma and liver stearate are higher than the other samples. The double bond sub-class 011 was lowest in liver and plasma, which illustrates a congruence between these two sites. The saturated fatty acid distribution in liver and plasma exhibited a further correspondence in that their second 001 sub-class had similar stearate percentages, which were also the highest observed in any site.

The liver is known to be the site of production of lipoprotein triglyceride which is then liberated into plasma (3). From this information it would be expected that plasma and liver triglyceride composition would be the same. It has been experimentally shown, using tracer techniques, that the liver has many pools of triglyceride (4,5) while the plasma also consists of several lipoprotein classes (6). Thus, it may be understandable that exact correspondence was not found in the total liver and plasma triglyceride extract.

In terms of tissue type it might be predicted that skeletal muscle and myocardium should be similar. However, there was a difference in fatty acid composition, myocardial triglyceride having more linoleic acid than skeletal muscle. The latter tissue also had more 011 and 111. There was actually much closer correspondence, with respect to double bond sub-classes, to the kidney. The saturated fatty acid percentage distribution in muscle and kidney also was more similar than muscle and myocardium, as was the case for the carbon number distribution. A point to consider is that skeletal muscle had adipose tissue interlarded between the fibers and so the muscle sample can not be considered as pure as the myocardial one. This might account for the fact that a divergence of values existed between these tissues.

The kidney triglyceride fatty acid exhibited the lowest oleic and highest palmitic and arachidonic acid content. On the other hand, the double bond sub-classes demonstrated no outlying values and the carbon number distribution was similar to skeletal muscle and plasma as well as the myocardium. The latter had the

TABLE IV

Triglyceride Carbon Number Distribution in Various Dog Tissues (Mole %)

Carbon number	Myocardium	Skeletal muscle	Liver	Kidney	Arterial plasma	Venous plasma	Omental adipose tissue	Subcutaneous adipose tissue	Pericardial adipose tissue	Perirenal adipose tissue
44	0.05	0.08	Trace	0.09	0.17	Trace	0.11	0.05	Trace	0.22
46	0.55	0.59	1.0	0.81	0.83	1.2	0.89	1.1	0.55	1.0
48	4.9	5.2	3.5	5.7	6.2	6.0	5.7	7.4	5.8	6.9
50	19.5	21.4	12.2	22.4	19.8	19.2	20.1	24.6	19.2	21.9
52	44.4	44.8	39.5	44.6	43.0	43.0	41.8	31.6	43.2	41.1
54	29.6	27.3	37.2	26.2	27.7	27.6	30.2	33.9	31.1	27.9
56	0.8	0.5	4.5	0.1	2.1	2.9	1.0	1.1	Trace	0.8

somewhat higher carbon number 54. The saturated fatty acid profile average showed that the kidney most resembled skeletal muscle. It is interesting to note that the perirenal adipose tissue carbon number distribution was quite similar to the whole kidney triglyceride profile.

When all sites are examined it can be observed that the double bond sub-classes 112 and 022 are remarkably constant, 112 averaging 10.5% and 022 averaging 3.3%. For four adipose tissues the 002 and 000 averaged 2.7% and 2.1% respectively, versus 5.2% and 2.7% for the other areas (excluding venous plasma). The latter was appreciably higher in both sub-classes and was thus not included in the calculated average. A similar comparison for 111 was 10.1% for depot fat and 13.6% for the rest, while 001 was 15.3% and 17.8%.

In the adipose tissue (1) the 022 sub-class contained the most stearate in the saturated fatty acid profile while in the tissues liver and plasma exhibited the greatest stearate in the second 001 band. Kaunitz et al. (7) reported data on human autopsy material. Their values indicated the 000 saturated fatty acids for heart, liver and perirenal adipose tissue to be quite similar when averaged, being 14:0, 12%; 16:0, 65%; 18:0, 21%.

The profile for 000 closely resembled the overall average for every site except subcutaneous and perirenal adipose tissue in the data on the dog. Therefore, if one wanted to use an average value the 000 distribution would be applicable as a good representative figure.

The literature has a dearth of data on triglyceride sub-class distribution in various organs. Kaunitz et al. (7) have results on human myocardium, liver and perirenal adipose tissue. These tissues have profiles which, on the average, are fairly similar. However, the liver showed more 011 and less 111 than the other two organs. The myocardial average was 000, 2.9%; 001, 16.9%; 011, 34.5%; 002, 4.4%; 111, 18.1%; 012, 14.9%; 112, 6.9%; 022, 1.4% and the liver was 000, 2.9%; 001, 19.1%; 011, 37.8%; 002, 3.7%; 111, 12.0%; 012, 17.3%; 112, 4.4%; 022, 1.6%. Slakey and Lands (8) have reported some data on rat liver after feeding rats Rockland Rat Diet for one week. They found 011, 13.8%; 012, 25.5%; 022, 11.3%. Their value for 011 was strikingly lower than any value in the dog or human, in which 011 was always the major sub-class. Their liver triglyceride fatty acid content was 18:1, 30% and 18:2, 25.1% and the lower oleate and higher linoleate undoubtedly contributed a shift in double bond sub-classes. Another point is that they found over 90% palmitate in their saturated fatty acid profile and practically no myristate.

That nutrition plays an important role can be seen in the report from Privett et al. (9) Rats on a fat free diet possessed similar triglyceride fatty acid content, e.g. liver 18:1 53.5%; kidney 18:1, 48.5%; plasma 18:1, 49.9%. When switched to a diet high in menhaden oil the values became liver 18:1, 25.3% and kidney 18:1, 34.5% while a diet with corn oil produced liver 18:1, 48.5% and kidney 18:1, 31.3%. Menhaden oil is relatively low in oleate and the liver oleate dropped lower than the kidney. On the converse, corn oil has a somewhat higher oleate and much higher linoleate and the liver 18:2 became 11.8% and the kidney rose to 34.5%. Thus, the dynamics of fatty acid incorporation into liver and kidney triglyceride showed marked differences which were apparently organ specific. It is interesting to note some of their double bond sub-class data, e.g., fat free diet: liver 001, 19.2%; 011, 59.8%; kidney 001, 29.0%; 011, 47.3%; menhaden oil: liver 001, 8.9%; 011, 15.7%; kidney 001, 28.7%; 011, 33.1%; corn oil: liver 001, 7.2%; 011, 36.4%; kidney 001, 10.1%; 011, 12.4%. Menhaden oil 18:1 was 20.6% and corn oil was 28.7%, values which were not very dissimilar. However, one can observe that the various diets produced quite different results in the sub-class examples selected.

It is clear from the results discussed that it will be necessary to not only determine the structure of the triglycerides in any particular site, but also to define the enzymes involved in their biosynthesis and how these activities are affected by nutritional events.

ACKNOWLEDGMENT

J. S. Steinberg and George Mathew gave technical assistance. Hahnemann Medical College Computer Center provided assistance (NIH Grant No. 1 SO1 FR05611). Supported in part by Grant No. AM 08475 from NIH.

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[Received November 12, 1968]

The Relation of Carnitine to the Formation of Phosphatidyl-β-Methylcholine by *Tenebrio molitor* L. Larvae[1]

L. L. BIEBER and R. E. MONROE, Biochemistry and Entomology Departments, Michigan State University, East Lansing, Michigan 48823

ABSTRACT

Results demonstrate that *Tenebrio molitor* larvae can incorporate β-methylcholine into their phospholipids and that β-methylcholine spares larval choline. Phosphatidyl-β-methylcholine is detected when larvae are reared on a diet in which choline is replaced by β-methylcholine. Results indicate that, in contrast to housefly and blowfly larvae, *Tenebrio* larvae do not metabolize carnitine to β-methylcholine. The same phospholipids were found with all rearing conditions. They were lecithin, sphingomyelin, phosphatidylethanolamine, phosphatidylserine and two others tentatively identified as cardiolipin and phosphatidylinositol. The ratio of lecithin to sphingomyelin and to the combined other phospholipids did not vary significantly, regardless of the rearing conditions. Larvae reared on diets lacking either choline or carnitine contained more lipid phosphorus per gram of tissue than those reared on a complete diet. The fatty acid composition of the neutral lipids was similar regardless of the rearing conditions. Oleic acid represented greater than 55% of the total fatty acids, and unsaturated fatty acids comprised 75-80% of the total. Palmitic acid was the predominant saturated fatty acid.

INTRODUCTION

Investigations by Fraenkel (1) demonstrated that carnitine and choline are required by *Tenebrio molitor* L. for growth. In contrast, certain dipteran larvae such as those of the blowfly *Phormia regina* L. (2,3), and housefly *Musca domestica* L. (4), and *Drosophila melanogaster* L. (5) can be reared on a diet in which choline is replaced by carnitine. When dietary choline is replaced by carnitine, little lipid bound choline is present in the phospholipids; rather, lipid bound β-methylcholine is found in the lecithin fraction of blowfly (6) and housefly (4) larvae. In addition to β-methylcholine, dimethylaminoethanol (4,7), dimethylaminoisopropanol (7) and trimethylaminoethylphosphonic acid (8) are incorporated into phospholipids in lieu of choline by housefly or blowfly larvae. Kamienski et al. (9) reported that the lecithin fraction, isolated from *Tenebrio* larvae reared on a normal diet, does not contain detectable amounts of phosphatidyl-β-methylcholine. Our studies confirm this observation; however, the results presented herein demonstrate that β-methylcholine is incorporated into phospholipids by *Tenebrio molitor* larvae.

MATERIALS AND METHODS

Preparation of Synthetic Diets

The synthetic diet used in these studies was similar to the one reported by Fraenkel et al. (10) with slight modification. The bulk nutrients of the diet were as follows: casein (Difco vitamin-free), 20%; glucose 30%; Wesson's salts 2%; and cholesterol (final concentration) 0.1%. These components, with the exception of the cholesterol, were ballmilled for 3 hr. The cholesterol was then added to the ground diet in dichloromethane and mixed until the solvent had evaporated. The standard vitamin mixture for 100 g diet was similar to that reported by Fraenkel (11): thiamine hydrochloride, 2.5 mg; riboflavin, 1.3 mg; niacin, 5.0 mg; pyridoxine-hydrochloride, 1.3 mg; calcium pantothenate, 2.5 mg; folic acid, 0.25 mg; biotin, 0.025 mg; and water 5.0 ml. The vitamins were dissolved in water by the addition of a few drops of ammonium hydroxide and then ground thoroughly into the prepared diet with a mortar and pestle. In the various experiments, 0.5 mg dl-carnitine, 0.5 mg γ-butyrobetaine, 50 mg choline or 50 mg dl-β-methylcholine, or both, as the chlorides were added to 100 g of diet. The diets were kept at -30 C until used.

Experimental Insects

The *Tenebrio molitor* larvae were obtained in the following manner. *Tenebrio* pupae were collected from a stock culture and placed into crystallizing dishes containing the synthetic diet but lacking choline and carnitine. These were placed into an incubator maintained at 30 C

[1]Journal Paper No. 4533, Michigan Agricultural Experiment Station, East Lansing, Michigan.

TABLE I

Phospholipid Content and Growth of *Tenebrio molitor* Larvae Reared on Diets Containing Carnitine or Choline Substitutes

Expt.	Additions to the diet	Micromoles of phospholipid per gram larvae	Per cent mortality	Larval weight mg/larvae	Total fatty acid in neutral lipids mg/g larvae
1	Choline + carnitine (control)	13.6	4	98	229
2	None	16.6	79	55	173
3	β-Methylcholine + carnitine	14.5	15	75	183
4	Choline + γ-butyrobetaine	16.9	68	64	167
5	Carnitine	---	>95	<5	---

and 75% ± 5% RH. The adults were allowed to emerge, mate and lay eggs on the diet; after hatching 25 larvae were placed into standard glass liquid scintillation vials containing 10 g of experimental diet. One hundred and fifty larvae were used for each test and rearing conditions were employed as reported above. When the larvae were near pupation (about 40 days after hatching), they were harvested, weighed live, frozen and stored at -30 C until analyzed.

Fatty Acid Analysis

The neutral lipids obtained from the column chromatographic fractionation described below were saponified for 1.5 hr in potassium hydroxide-ethanol (10:90 v/v) under nitrogen. The mixture was diluted with water and extracted with ethyl ether. The aqueous solution was then acidified with concentrated hydrochloric acid and quantitatively extracted with ether. After drying over anhydrous sodium sulfate, the ether was evaporated in vacuo and the free fatty acids weighed.

The fatty acids were methylated as reported by Metcalf and Schmitz (12), and the methyl esters subjected to gas liquid chromatography. Detailed column conditions are reported under Results; all retention times were compared to standard fatty acid mixtures C, D, E and F, as obtained from W. H. Goldwater (National Institutes of Health, Bethesda, Md). Relative per cents of fatty acids present were calculated by disc integration.

Isolation, Separation and Identification of Phospholipids

The lipids were extracted from larvae by homogenizing twice for 2 min at room temperature in chloroform-methanol (1:1) containing 0.02% butylhydroxytoluene. The ratio solvent-

TABLE II

Composition and Amount of Phospholipids Derived From *Tenebrio molitor* Larvae Reared on Various Diets

Compounds added to diet	Per cent of the total lipid phosphorus in the fraction	Lipids detected in the fraction[a]
Choline + carnitine (control)	56	PE, PS, PI, Cardiolipin?
	36	PC
	6	SP
None	57	PE, PS, PI, Cardiolipin?
	32	PC
	11	SP
Carnitine + β-methylcholine	60	PE, PS, PI, and Cardiolipin?
	32	PC, PMC
	8	SP
Choline + γ-butyrobetaine	59	PE, PS, PI, and Cardiolipin?
	36	PC
	5	SP

[a]PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SP, sphingomyelin; PC, phosphatidylcholine; PMC, phosphatidyl-β-methylcholine.

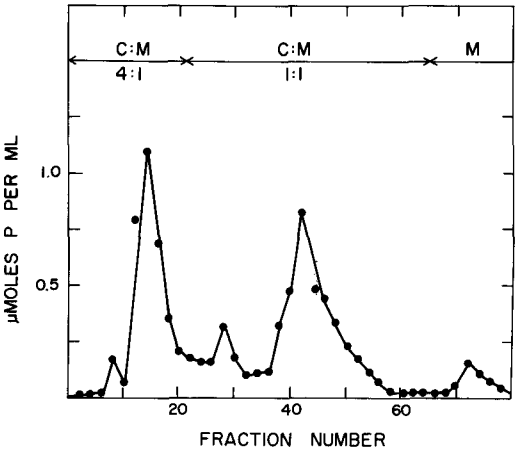

FIG. 1. Separation of *Tenebrio molitor* phospholipids by chromatography on silicic acid. Phosphorus analyses were done on 0.1 ml aliquots of the even-numbered fractions. Fractions 1-31, 32-56, and 57-80 were combined and analyzed as described in the Methods. C, chloroform; M, methanol.

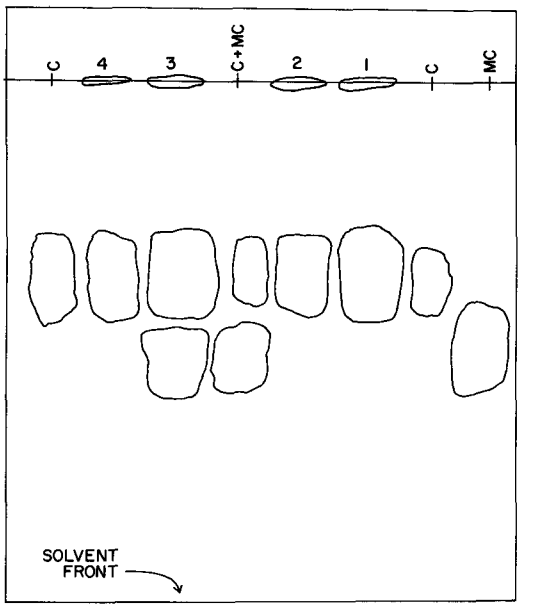

FIG. 2. Representation of a paper chromatogram of the acid hydrolysates of the lecithin fractions obtained from larvae reared in the presence of various dietary choline or carnitine substitutes. Acid hydrolysates of the materials equivalent to those found in fractions 32-56 of Figure 1 were chromatographed on paper in 95% ethanol-conc. ammonium hydroxide. Choline and β-methylcholine were detected by spraying the chromatograms with Dragendorf's reagent. Numbers 1, 2, 3 and 4 are from the lecithin fractions (phosphatidylcholine and phosphatidyl-β-methylcholine) described for Experiments 1, 2, 3 and 4 of Table I. C, choline chloride; MC, β-methylcholine chloride.

tissue (v/w) was 15 for each extraction. The combined organic extracts were washed as described previously (13). The lipids in chloroform were applied to a stepdown column containing 75 ml of BioSil A as described elsewhere (8). The columns were eluted successively with 100 ml of chloroform, 150 ml of acetone, 250 ml of chloroform-methanol (4:1), 500 ml of chloroform-methanol (1:1) and 150 ml of methanol. The combined chloroform and acetone eluate represented the neutral lipid fraction. The solvents contained 0.02% butylhydroxytoluene (w/v), and phosphate was determined by the method of Bartlett (14). The presence of phospholipid bound β-methylcholine, choline, ethanolamine and serine was investigated by chromatographing the acid hydrolysates on paper as described previously (6,15)

Thin layer chromatography was carried out on Brinkman Silica Gel F 254 precoated plates using the following solvent systems: chloroform-methanol-H_2O (65:35:4) and chloroform-methanol-diisobutylketone-glacial acetic acid-water (45:15:30:20:4). Reference lecithin, sphingomyelin, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine were prepared from a lipid extract of beef liver by column chromatography on silicic acid, followed by chromatography on DEAE cellulose. Lysolecithin was prepared from lecithin by treatment with phospholipase A. The chloroform soluble extract of the phospholipase A reaction mix was resolved by chromatography on silicic acid. Cardiolipin was purchased from Pierce Biochemicals. Lipids on the thin layer chromatograms were detected using iodine vapors. Free as well as bound choline and β methylcholine were detected using iodine vapors and Dragendorf's reagent.

RESULTS

Phospholipid Content and Growth Response of Larvae Reared on Diets Containing Choline and Carnitine Substitutes

The data in Table I demonstrate that the mortality of *Tenebrio molitor* larvae is slightly greater when larvae are reared on a diet in which choline is replaced by β-methylcholine. In agreement with the results of Fraenkel (1), the mortality of larvae is high when dietary carnitine is replaced by γ-butyrobetaine or when choline and carnitine are omitted. When larvae are reared on a carnitine-supplemented diet containing very little choline (Fison rather than Difco casein was used), growth is greatly

TABLE III

Gas Liquid Chromatographic Analysis of the Fatty Acid Methyl Esters of Neutral Lipids Isolated From *Tenebrio molitor* Larvae Reared on Diets Containing Carnitine or Choline Substitutes

Test diet	Relative per cent fatty acids[a]						
	C-12	C-14	C-16	C-16:1	C-18	C-18:1	C-18:2
With choline and dl-carnitine (control)	Trace	9.2	16.9	5.0	Trace	58.7	10.1
No choline or dl-carnitine	Trace	5.0	18.4	3.5	Trace	63.7	9.5
With β-methylcholine and dl-carnitine	Trace	2.8	15.1	4.7	Trace	75.8	1.7
With choline and γ-butyrobetaine	Trace	4.1	19.7	4.4	Trace	62.4	9.4

[a]Column: 15% diethylene glycol succinate on 100-120 Gas-Chrom Q, nitrogen 53.1 x ml/min, column 200 C (stainless steel 6 ft x 4 mm I.D.), vaporizer 240 C, hydrogen flame detector 235 C.

retarded and the mortality is very high as shown in Experiment 5 of Table I. Larvae that were reared using conditions of high mortality weighed less than the controls and had less neutral lipids, but contained 2 to 2.5 μmoles more phospholipid per gram of tissue.

Phospholipid Composition of Larvae Reared on Diets Containing Choline and Carnitine Substitutes

Lipids were isolated from the larvae described in Experiments 1-4 of Table I and the phospholipids resolved by column chromatography on silicic acid. A typical lipid-phosphorus elution profile is shown in Figure 1. These results were obtained from larvae reared on the carnitine and β-methylcholine diet. The phosphorus profiles of lipids isolated from larvae reared on the other diets were similar except that the lecithin fractions, tubes 36-60, were not as broad. When the phospholipids from Experiments 1, 2 and 4 of Table I were separated, the lecithin fractions were eluted in 16-18 fractions rather than the 25 fractions shown in Figure 1. The broad lecithin peak shown in Figure 1 is most likely caused by the presence of phosphatidyl-β-methylcholine. Phosphatidyl-β-methylcholine is eluted from silicic acid slightly ahead of, but overlapping, phosphatidylcholine (6).

Thin layer chromatography of the material in fractions 1-31 of Figure 1 indicated the presence of phosphatidylethanolamine, phosphatidylserine, and 2 ninhydrin-negative materials with R_f's of phosphatidylinositol and cardiolipin. Paper chromatography of the acid hydrolysates of these lipids demonstrated the presence of two ninhydrin-positive materials which had R_f's identical to ethanolamine and serine. As summarized in Table II, identical results were obtained with the phospholipids from larvae described in Experiments 1, 2 and 4 of Table I.

Thin layer chromatography of the materials present in the peaks equivalent to fractions 32-56 of Figure 1 indicated the presence of phosphatidylcholine. One of the lecithin fractions, the one isolated from larvae reared on a diet containing added carnitine and β-methylcholine, contained a Dragendorf reagent-positive phospholipid that had an R_f slightly greater than phosphatidylcholine. Sellers (16) has shown that the phosphatidyl-β-methylcholine, isolated from housefly larvae, has an R_f slightly greater than phosphatidylcholine when chromatographed on Brinkman precoated Silica plates using the solvent system, chloroform-methanol-water (65:35:4). The presence of β-methylcholine was confirmed by chromatographing the acid hydrolysates on paper in ethanol ammonium hydroxide (95:5). As illustrated in Figure 2, phosphatidyl-β-methylcholine is present in the lecithin fraction isolated from larvae that were reared on a diet free of added choline but containing carnitine and β-methylcholine. Besides having an R_f of β-methylcholine, the color produced by Dragendorf s reagent was orange, a color identical to that of the β-methylcholine standard, rather than the reddish purple color given by choline. β-Methylcholine was not detected in the lecithin fractions derived from larvae reared in the absence of added β-methylcholine even though hydrolyzed phospholipid equivalent to as much as 20 μmoles of phosphate was applied as a single spot on the paper chromatogram. If the other lecithin fractions contained as little as 2-3% phosphatidyl-β-methylcholine, it would have been detected. This argument is not valid

for the larvae described in Experiment 5, Table I, since less lipid was available. In Experiment 5, *Tenebrio* larvae were reared identical to those in Experiments 1-4 except that casein obtained from Fison's Pharmaceutical Ltd., Loughborough, Leichestershire, Great Britain, was used instead of Difco casein. Fison casein was only used in Experiment 5. The choline content of Fison casein is approximately 1/5 that of Difco casein as determined by microbiological assay. Most of the larvae from Experiment 5 died; however, after 8 weeks, 90 mg of live larvae were collected. They were homogenized in 20 ml of chloroform-methanol (1:1), followed by homogenization in 20 ml of chloroform-methanol-water (5:5:1). The combined organic extracts were taken to dryness without washing. This extract should contain free as well as lipid bound choline, β-methylcholine and carnitine. Paper chromatography of the hydrolysate demonstrated the presence of choline but not β-methylcholine, indicating that carnitine was not converted to β-methylcholine.

Thin layer chromatography of the material eluted by methanol from silicic acid, fractions equivalent to tubes 57-80 of Figure 1, demonstrated the presence of a material that had an R_f identical to beef liver sphingomyelin. Paper chromatography of the hydrochloric acid hydrolysate of the sphingomyelin-containing fractions demonstrated the presence of choline and the absence of ethanolamine and serine. A small amount of a material having the R_f and Dragendorf color of β-methylcholine was detected in the acid hydrolysate of the sphingomyelin fraction isolated from larvae reared in the presence of carnitine and β-methylcholine. The identity of this material as a β-methylcholine-containing sphingolipid must remain tentative until further characterization is completed. Lyso-phosphatidyl-β-methylcholine, although not detected in the sample by thin layer chromatography, if present, would occur in this fraction.

Fatty Acid Composition of the Neutral Lipids

The fatty acid composition of the neutral lipids isolated from larvae reared on the various diets is shown in Table III. Unsaturated fatty acids comprise 75-80% of the total. The principal fatty acid in the neutral lipids is oleic acid which represents greater than 55% of the total fatty acids. Palmitic acid is the principal saturated fatty acid.

DISCUSSION

The results demonstrate that *Tenebrio molitor* larvae can synthesize phosphatidyl-β-methylcholine. The choline-sparing action of β-methylcholine, but not carnitine, indicates that larvae do not decarboxylate carnitine. The requirement of both carnitine and choline for optimal larval growth in *Tenebrio*, reported by Fraenkel (1) and confirmed by us, is in accord with this conclusion. Blowfly (6,15) and housefly (4,16) larvae are different in that they can convert carnitine to β-methylcholine; however, adult houseflies are like *Tenebrio* in that they decarboxylate little, if any, carnitine (4). One apparent difference between the composition of the Tenebrio lecithin fraction and the housefly or blowfly lecithin fractions is that much more β-methylcholine is found in the lecithin fraction of the dipterans. Greater than 90% of the lecithin fraction can be phosphatidyl-β-methylcholine in housefly (16) and blowfly larvae (6,15). Although quantitative studies on phosphatidyl-β-methylcholine were not done, the small size of the β-methylcholine spot on paper chromatograms compared to the area of the choline spot demonstrated more choline was present than β-methylcholine.

The results demonstrate that the ratio of lecithin to the other phospholipids in *Tenebrio* is constant over a wide range of growth conditions. The lecithin content varied from 32-36% of the total lipid phosphorus. In the experiment in which the choline content of the lecithin fraction was diminished, another quaternary ammonium containing alcohol, β-methylcholine, was found and the amount of lecithin remained constant. There was a small variation in the micro moles of phospholipid phosphorus per gram of larvae. This value was least (13.6 μmoles) for the heavier and healthier larvae and greatest (16.9 μmoles) for larvae reared under conditions of high mortality. Such results indicate that there is a minimum amount of phospholipid required by the larvae; such a minimum requirement is consistent with phospholipid involvement in membranes. A plausible interpretation of our results is that when larvae are reared on diets containing traces of choline (microbiological assay demonstrated small amounts of choline in the casein), few larvae grew because of insufficient choline for one or more vital functions such as membrane formation; however, when a choline substitute was present, better larval growth occurred because the substitute could replace choline in membrane formation. This interpretation is not inconsistent with the results reported by Geer (17) for *Drosophila* larvae and adults. He found that β-methylcholine is an adequate substitute for dietary choline during larval development, but the resulting adult males are sterile because of immobile sperm.

The phospholipid composition described in these experiments was similar to that reported by Kamienski et al. (9). They reported the presence of phosphatidylethanolamine, phosphatidylcholine, sphingomyelin, phosphatidylinositol and two or three unknowns. Kamienski et al. (9) did not detect phosphatidylserine in their preparations; however, the combination of comparative thin layer chromatography and identification of the acid hydrolysis products demonstrated the presence of small amounts of phosphatidylserine in *Tenebrio molitor* lipids.

The fatty acid composition of the neutral lipids was not affected by replacing dietary choline with β-methylcholine or by replacing carnitine with γ-butyrobetaine. Such results indicate that carnitine exerts little, if any, control on the fatty acid composition; however, as shown in Table I, larvae reared in the absence of added choline or added carnitine contained considerably less neutral lipid fatty acids per gram of larvae. Such results indicate that carnitine plus choline exerts a positive effect on the synthesis or storage of larval fatty acids. Carnitine participates in the transport of fatty acids across certain membranes (18); this transport role of carnitine could be involved in formation of depot lipids, triglycerides, as well as in the oxidation of fatty acids. The very high concentration of neutral lipids in the larvae most likely is an energy reserve that can be used during metamorphosis.

ACKNOWLEDGMENTS

Supported by Grant 5 RO1 AM10514 from the Division of Arthritis and Metabolic Diseases, United States Public Health Service. Technical assistance by Donna Peterson.

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[Received October 16, 1968]

SHORT COMMUNICATIONS

The Lipids of *Lepisma saccharina* L.

The lipids of insects generally show a marked similarity in composition to those obtained from higher animals, and existing knowledge reveals that their metabolism is also quite similar (6,8). However, there are some exceptions, and Fast (3) has suggested that those organisms which show marked differences in lipid composition may be productively utilized in correlating functional mechanisms with molecular structure and composition, particularly with regard to membrane functions. The present communication describes the composition of the major lipid components of the silverfish, *Lepisma saccharina* L. This wingless species, a member of the order Thysanura, represents one of the most primitive forms of insect life. Its diet consists of carbohydrate materials mainly paper and book binding glue. In view of its phylogenetic primitiveness and limited diet, it was of interest to determine the composition of its body lipids and compare them with those isolated from representatives of higher species.

The experimental specimens (average body length 12 mm) were collected over a period and preserved in chloroform at -20 C. The lipids of pooled samples were extracted and washed according to the method of Folch et al. (5). The extracted lipids were dried under nitrogen and following determination of their weight they were redissolved in redistilled diethyl ether for further analysis.

The various lipid classes were separated by thin layer chromatography using a system (9) whereby the phospholipids remained at the origin. The various neutral lipid classes were identified by comparing their Rf values with authentic standards (Applied Science Labs, State College, Pa.) chromatographed on the same plates. The Rf values of the neutral lipid classes were: monoglycerides 0.1, diglycerides 0.25, cholesterol 0.31, free fatty acids 0.54, triglycerides 0.75 and cholesterol esters 0.91, respectively. The percentage distribution of these lipid classes was determined by densitometry according to the procedure of Privett et al. (13).

The phospholipids were separated by the modified (12) two dimensional chromatographic system developed by Rouser et al. (14). The separated lipids were identified by cochromatography with standard mixtures of purified phospholipids (Applied Science) and using specific spray reagents (16). Lipid phosphorus was quantified by the procedure of Rouser et al. (15).

Fatty acid methyl esters were prepared according to the method of Metcalfe and Schmitz (11). The composition of this mixture was determined by gas liquid chromatography. A Barber-Colman Model 5000 gas chromatograph equipped with a hydrogen flame detector, and containing a 6 ft 4 mm I.D. column packed with 10% diethylene glycol adipate on Chromosorb W (Analabs, Hamden, Conn.) was used. This was temperature programmed from 120-195 C at 10° per minute during analyses, and argon (14 lb/in.^{2}) was used as carrier gas. Quantification was obtained by triangulation. The fatty acids were identified by comparing their retention times to those of authentic standards (Applied Science, State College, Pa.) and by plotting the retention times against carbon numbers.

The composition of the lipids, which composed approximately 8% of the body weight of the specimens, is shown in Table I. The triglycerides were the dominant class of lipids present, which is in accord with data from other species (7,9) However, the rather large quantity of cholesterol esters occurring in these lipids is very unusual. It is difficult to explain this observation since little is known of the metabolism of this species. Possibly these sterol

TABLE I

Composition of the Lipids of *Lepisma saccharins* L.

Lipid class	Percentage distribution Range
Phospholipids	3.2 - 5.5
Monoglycerides	Trace - 1.0
Diglycerides	1.6 - 2.0
Cholesterol	3.7 - 4.6
Free Fatty Acids	1.5 - 2.6
Triglycerides	56.0 - 68.0
Cholesterol ester	20.0 - 25.0

TABLE II

Composition of the Phospholipids of *Lepisma saccharina* L.

Phospholipid class	Percentage distribution
Lysophosphatides	4.0
Sphingomyelin	10.4
Phosphatidylcholine	36.3
Phosphatidylinositol	2.6
Phosphatidyl serine	1.1
Phosphatidylethanolamine	37.7
Cerebrosides	7.8
Cardiolipid	0.1

TABLE III

Fatty Acid Composition of the Total Lipids of *Lepisma saccharina* L.

Fatty acid Carbon no.	Per cent by weight	Fatty acid carbon no.	Per cent by weight
6:0	Trace	16:0	20.50
8:0	0.31	16:1	5.10
10:0	0.39	16:2	0.16
12:0	0.51	17:0	1.68
12:1	0.02	17:1	0.03
13:0	0.08	18:0	9.14
13:1	Trace	18:1	47.00
14:Br.	Trace	18:2	10.40
14:0	2.70	18:3	0.12
14:1	0.18	20:0	0.13
14:2	0.12	20:2	Trace
15:0	0.83	20:4	Trace
15:1	0.08	22:0	Trace
16:Br.	0.08	24:0	Trace

esters may be components of the silver scales with which the body of this insect is covered. The phospholipids show the same qualitative composition as reported for other insect species (1-3). Quantitatively they display differences which may be significant. With the exception of the dipterous insects and some species of the family Aphidae (3), phosphatidyl choline is the dominant phospholipid class occurring in insects. In *Lepisma* both the ethanolamine and choline phosphoglycerides occur in roughly equal amounts. The reason once again is obscure and warrants investigation. Similar proportions have been found in the diperous family Cecidomyiidae (3).

Also noteworthy is a large quantity of cerebrosides occurring in this species. This class has not been reported in other insect species and it may reflect a species difference.

Forth discrete peaks appeared on the chromatogram following analysis by gas chromatography and those which were identified are presented in Table III. Qualitatively this species contains a wide array of fatty acids, however quantitatively, myristic (C14:0) palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1) and linoleic (C18:2) comprise 95% of the total fatty acids, which is not abnormal for insect lipids. The very high quantities of oleic and linoleic is commonly observed in widely unrelated insects (4).

It thus seems that, despite wide taxonomical phylogenetic differences, insect species (and also vertebrates) possess a rather constant lipid composition, suggesting a rather similar biosynthetic capacity.

JOHN E. KINSELLA
Department of Food Science
Cornell University
Ithaca, New York 14850

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[Received October 28, 1968]

A Simplified Method for Extracting Lipids From Large Quantities of Tissue Abundant in Water

The method described by Bligh and Dyer (1) for extracting lipids from vertebrate tissues is commonly used in our laboratory for invertebrate tissues. Usually small corrections are necessary, depending on the water content. Since the amount of chloroform and methanol is calculated in relation to the fresh (wet) weight of the animals, the required quantities of these solvents are quite considerable if this extraction procedure is applied to a large quantity of tissue with high water contents.

A modification of this procedure permits an

TABLE III

Fatty Acid Composition of the Total Lipids of *Lepisma saccharina* L.

Fatty acid Carbon no.	Per cent by weight	Fatty acid carbon no.	Per cent by weight
6:0	Trace	16:0	20.50
8:0	0.31	16:1	5.10
10:0	0.39	16:2	0.16
12:0	0.51	17:0	1.68
12:1	0.02	17:1	0.03
13:0	0.08	18:0	9.14
13:1	Trace	18:1	47.00
14:Br.	Trace	18:2	10.40
14:0	2.70	18:3	0.12
14:1	0.18	20:0	0.13
14:2	0.12	20:2	Trace
15:0	0.83	20:4	Trace
15:1	0.08	22:0	Trace
16:Br.	0.08	24:0	Trace

esters may be components of the silver scales with which the body of this insect is covered. The phospholipids show the same qualitative composition as reported for other insect species (1-3). Quantitatively they display differences which may be significant. With the exception of the dipterous insects and some species of the family Aphidae (3), phosphatidyl choline is the dominant phospholipid class occurring in insects. In *Lepisma* both the ethanolamine and choline phosphoglycerides occur in roughly equal amounts. The reason once again is obscure and warrants investigation. Similar proportions have been found in the diperous family Cecidomyiidae (3).

Also noteworthy is a large quantity of cerebrosides occurring in this species. This class has not been reported in other insect species and it may reflect a species difference.

Forth discrete peaks appeared on the chromatogram following analysis by gas chromatography and those which were identified are presented in Table III. Qualitatively this species contains a wide array of fatty acids, however quantitatively, myristic (C14:0) palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1) and linoleic (C18:2) comprise 95% of the total fatty acids, which is not abnormal for insect lipids. The very high quantities of oleic and linoleic is commonly observed in widely unrelated insects (4).

It thus seems that, despite wide taxonomical phylogenetic differences, insect species (and also vertebrates) possess a rather constant lipid composition, suggesting a rather similar biosynthetic capacity.

JOHN E. KINSELLA
Department of Food Science
Cornell University
Ithaca, New York 14850

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A modification of this procedure permits an

easier as well as a more economical extraction of lipids. After crushing or grinding the tissue and allowing the slurry to settle, the tissue water is decanted into a separate beaker and its volume measured. The residual tissue slurry is weighed and mixed thoroughly in a separatory funnel first with methanol then with chloroform in the proportion, tissue residue-methanol-chloroform (0.8:2.0:1.0 w:v:v). After adding once more the same quantity of chloroform to the mixture, the tissue water is added, and after mixing this water fraction with the homogenate a biphasic system should be formed. If necessary small portions of distilled water can be added until two phases are obtained.

From this point on the procedure is identical to that of Bligh and Dyer. This modification is advantageous only for those tissues which are abundant in water content.

D. J. VAN DER HORST
A. H. VAN GENNIP
P. A. VOOGT
Laboratory of Chemical Animal Physiology,
University of Utrecht,
40, Jan van Galenstraat,
Utrecht, The Netherlands

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[Received October 14, 1968]

Pyrolysis–Gas Chromatography of Phosphoglycerides: a Mass Spectral Study of the Products

During attempts to volatilize phosphoglycerides for analysis by gas liquid chromatography (GLC), it was observed that when short, nonpolar columns were employed at high temperatures, components were eluted within a few minutes. Preliminary investigations showed that the eluates were not phosphoglycerides. Since these components were formed only at high temperatures of both the injection port (over 300 C) and column (over 200 C) it was concluded that they were probably the products of pyrolysis of the phosphoglycerides. The peaks obtained were found to have retention times coincident with those from the appropriate 1,2 and 1,3 diglycerides injected under the same conditions. Thus, dipalmitoyl phosphatidyl choline gave rise to a single peak with a retention time the same as that obtained from dipalmitin. This observation was confirmed for dilauroyl dimyristoyl, dipalmitoyl and distearoyl phosphatidyl cholines and for dipalmitoyl phosphatidyl ethanolamine (Sigma) Chemical Corporation and Mann Research Laboratories). Phosphatidyl serine (ex. bovine brain, Applied Science Labs, Inc.) and egg lecithin (laboratory preparation) gave rise to several overlapping peaks, the major one having a retention time close to that of distearin or 1,1-stearylolein, or both. At the time this study was being carried out, Kuksis et al. (1) reported that, when total serum lipids were gas chromatographed using a specially modified injector port at temperatures over 300 C, the serum lecithins gave rise to a series of overlapping peaks. They tentatively identified these peaks as the fatty acid diesters of propenediol, pyrolysis products of the lecithins. We now report the results of our study of phosphoglyceride pyrolysis using gas chromatography-mass spectrometry (GLC-MS).

Mass spectra were determined with a Perkin Elmer Hitachi RMU 6E single focusing mass spectrometer equipped with a gas chromatographic inlet system. The helium separator was maintained at 290 C as was the ion source. The heated transfer line from the GLC was kept at 325 C. The ionization potential was 22 ev and ionizing current was 55 a. Spectra were recorded in 4-10 sec to m/e 600 at the apex of the GLC peak (1-50 μ g of material), as determined by the continuous record produced by the total ionization monitor. The gas chromatograph employed a stainless steel column 2 ft x 1/8 in. packed with 5% SE30 coated on 60-80 mesh Chromosorb W(aw), with a flow rate of 25 ml/min. Approximately 85% of the effluent was diverted to the mass spectrometer. Elution of individual compounds was achieved either by operating isothermally at 280 C or by programming the column temperature from 200 C to 360 C at 12 C/min. The injector temperature was 350-400 C and the detector was at 360 C. The GLC column, the helium separator and the heated inlet line were silanized with Silyl 8 column conditioning agent. No major modifications were made to the gas chromatograph, but in order to obtain consistent results on-column injection was found to be essential. This was

easier as well as a more economical extraction of lipids. After crushing or grinding the tissue and allowing the slurry to settle, the tissue water is decanted into a separate beaker and its volume measured. The residual tissue slurry is weighed and mixed thoroughly in a separatory funnel first with methanol then with chloroform in the proportion, tissue residue-methanol-chloroform (0.8:2.0:1.0 w:v:v). After adding once more the same quantity of chloroform to the mixture, the tissue water is added, and after mixing this water fraction with the homogenate a biphasic system should be formed. If necessary small portions of distilled water can be added until two phases are obtained.

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D. J. VAN DER HORST
A. H. VAN GENNIP
P. A. VOOGT
Laboratory of Chemical Animal Physiology,
University of Utrecht,
40, Jan van Galenstraat,
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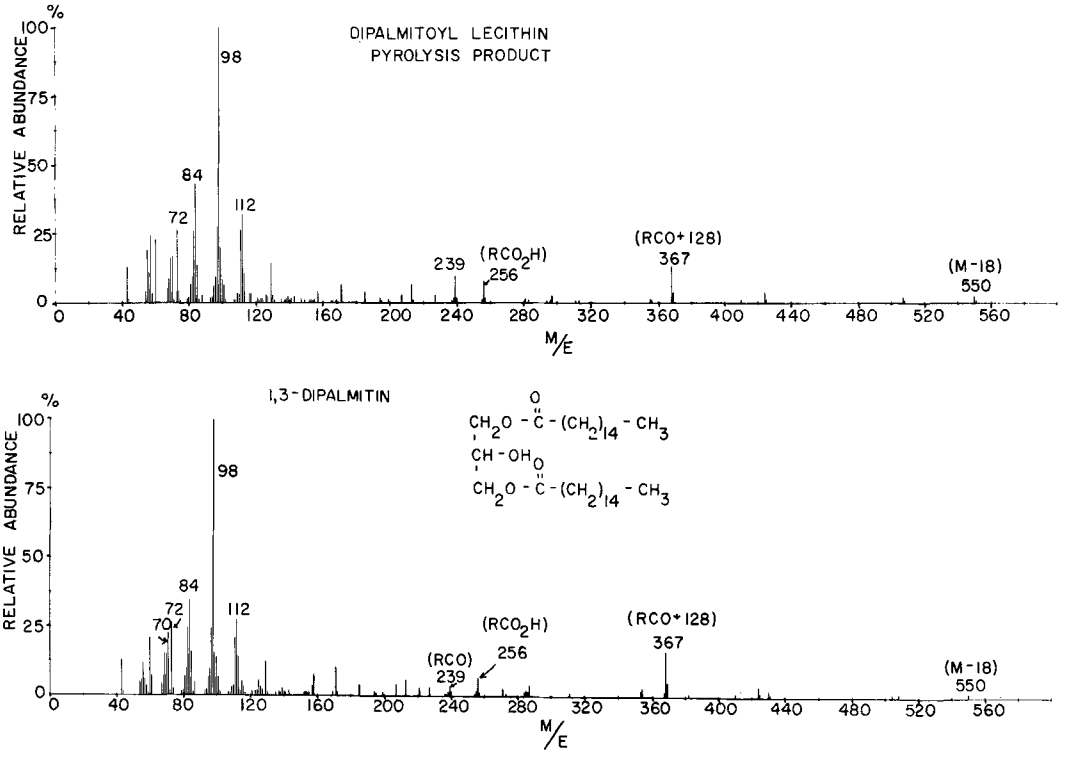

FIG. 1. Mass spectrum of the pyrolysis product of dipalmitoyl phosphatidyl choline and 1,3 dipalmitin. Introduced via the gas chromatograph.

achieved by projecting the column back through the injection port and filling the first two inches with glass wool. Injection was then made onto the glass wool packing. It was also necessary to precondition the column each day by several injections of a sample until a constant response was obtained.

The mass spectrum of the single peak obtained from the injection of dipalmitoyl phosphatidyl choline onto a short GLC column is shown in Figure 1. This peak is identical both in retention time and mass spectrum to that obtained from the injection of 1,2- or 1,3-dipalmitin (Fig. 1). Introduction of the diglycerides via GLC and by the direct inlet probe gave identical mass spectra. However, mass spectra were obtained for phosphoglycerides only under the GLC conditions described and not by the direct inlet probe, even when a temperature of 450 C was attained.

The mass spectra obtained exhibited a complete series of ions resulting from the cleavage of the fatty acid moiety and numerous hydrocarbon fragments similar to those described by Barber et al. (2). Other ions corresponding to RCO, RCO_2H and M-RCO_2H were also observed. Mass spectra obtained for phosphoglycerides, diglycerides and 1,2- propylene glycol dipalmitin and distearin all showed the formation of the base peak at m/e = 98. High resolution mass measurements indicated that this ion had a molecular formula corresponding to $C_6H_{10}O^+$ (calculated mass, 98.073164; found 98.073131).

This fragment probably has the structure of a cyclic ion, and may be formed from the 6,7 cleavage of the acyl moiety as suggested by Ryhage and Stenhagen (3). Support for the origin of this ion is also provided by the appearance of a metastable peak corresponding to the transition of m/e = 239 (the acyl moiety of $C_{16}H_{31}O$) to m/e = 98. Another structure for an m/e 98 ion, derived from the glycerol backbone and alpha cleavage of the acyl moiety of monoglycerides, with the formula $C_5H_6O_2^+$ (calculated 98,03678), was postulated by Johnson and Holman (4) to explain the presence of this ion in the mass spectra reported by them.

In the present study, the m/e = 98 peak was the base peak in spectra of diglycerides obtained via the GLC at an ionizing potential of 22 ev. This peak was reduced in size but was still a major ion when the sample was intro-

duced by direct inlet. At a higher voltage (70 ev), when there was a greater degree of fragmentation, the intensity of this ion was reduced (25-35%). The base peak observed at 70 ev was m/e = 57 ($C_4H_9^+$). The pyrolysis GLC-MS of dipalmitoyl phosphatidyl ethanolamine gave rise to the same mass spectrum observed from dipalmitin and dipalmitoyl lecithin. The same generalized fragmentations were observed from the GLC-MS of 1,3-dilaurin, 1,3-dimyristin, 1,3-distearin and 1-palmitoyl-3-stearin. In no case was a molecular ion observed; only the ion corresponding to M-18 was found.

One would expect that lysophosphatidyl compounds would also pyrolyze to the corresponding monoglyceride moiety. Accordingly, 1 monopalmitoyl phosphatidyl choline, when subjected to the pyrolysis conditions previously outlined, yielded a product with the same mass spectrum as that obtained from 1-monopalmitin. These spectra exhibited the same base peak at m/e 98. The same characteristic spectra were also obtained from a homologous series of 1-monoglycerides (C_{12} - C_{18}) including 1-monoolein. Under identical GLC conditions no pyrolysis of sphingomyelin was observed.

Since the C-O bond in the phosphate ester is somewhat weaker (85 Kcal/mole) than the O-P bond (95 Kcal/mole), its preferential destruction on pyrolysis of phosphoglycerides is to be expected. These pyrolysis products would give rise to the same compounds as the corresponding dehydrated diglycerides, namely the diacyl esters of propenediol.

E. G. PERKINS
PATRICIA V. JOHNSTON
The Burnsides Research Laboratory
Department of Food Science
University of Illinois
Urbana, Illinois 61801

ACKNOWLEDGMENT

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[Received January 2, 1969]

The Polyunsaturated Alcohols in Wax Esters of Fish Roe

Past investigations of wax esters from marine sources indicated that unsaturation of their constituent alcohols and acids is almost wholly confined to components having only one double bond. However, fatty acids of wax esters from mullet (*Mugil cephalus*) roe were found to be equally or even more highly unsaturated than those of the glycerides in roe and body oil (1). Other reports based on tentative identifications by GLC showed the presence of polyunsaturated alcohols in wax esters of marine animals (2,3). Unequivocal identification of minor components is desirable in view of the well known complexity of fish lipids in regard to polyunsaturation, positions of double bonds, branched or odd-numbered compounds. We report here results of analyses in which the polyunsaturated alcohols were isolated as acetates and their structures were determined by chemical degradation.

Wax esters from the roe of gouramis (*Trichogaster cosby*), a tropical fresh water fish, and of mullets (*Mugil cephalus*) from the coastal waters of the Gulf of Mexico were investigated. The lipids of their eggs contain > 70% wax esters. To obtain a concentrate of unsaturated wax esters, the lipid material was crystallized from hexane. The more unsaturated portion of the wax esters from the mother liquor was isolated from other lipids by column chromatography on SiO_2 and transesterified by sodium methylate in methanol. Alcohols were then separated from methyl esters by chromatography on SiO_2 and unsaturated alcohols were enriched in the mother liquor by crystallization from hexane and were acetylated. The alcohol acetates were then fractionated by GLC on cyclohexaamylose valerate. Fractions of uniform chain length were collected and subfractionated by GLC on ethylene glycol succinate (4).

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E. G. PERKINS
PATRICIA V. JOHNSTON
The Burnsides Research Laboratory
Department of Food Science
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Urbana, Illinois 61801

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These analytical procedures permitted tenta-

TABLE I

Constituents of Wax Esters From Gourami Egg Oil

Structure	Alcohols		Acids[a]	
	%	$ECL_{alc\text{-}ac}$[b]	%	$ECL_{Me\ ester}$[b]
14:0	2.7	(14)	0.9	(14)
16:0	45.0	(16)	1.9	(16)
16:1	8.8	16.5	10.8	16.7
18:0	6.8	(18)	0.5	(18)
18:1	30.1	18.5	51.0	18.6
9,12-18:2	1.3	19.3	13.5	19.6
9,12,15-18:3	0.8	20.6	4.6	20.7
20:0	0.1	(20)	<0.1	(20)
20:1	2.0	20.7	<0.1	20.6
11,14-20:2	1.1	21.6	<0.1	21.5
8,11,14-20:3	<0.1	22.2	0.3	22.1
11,14,17-20:3	0.5	22.6	<0.1	22.5
5, 8,11,14-20:4	<0.1	22.9	1.7	22.8
8,11,14,17-20:4	0.2	23.3	1.0	23.3
5, 8,11,14,17-20:5	0.1	23.9	1.4	23.9
7,10,13,16,19-22:5	0.3	25.7	3.7	25.9
4,7,10,13,16,19-22:6	0.1	26.4	6.8	26.9

[a]Incomplete listing.

[b]Equivalent chain length, i.e., retention time in reference to saturated compounds of the same type and chain length, on ethylene glycol succinate at 180 C.

tive identifications. They were repeated on a larger scale to afford samples sufficient for determination of structures. Ozonization and subsequent hydrogenation of ozonides was carried out with the individual compounds or mixtures of isomers (4). The fragments obtained from the alcohol acetates were identified by GLC using authentic aldehyde-acetates as reference compounds. The structures determined by ozonolysis confirmed the tentative identifications of alcohol acetates by GLC. Quantifications were checked by hydrogenation and subsequent GLC. All polyunsaturated structures of alcohols were also found among the acids (Table I). The same identity of structures was encountered with the polyunsaturated alcohols and acids of wax esters from mullet roe.

The structural relationships suggest that the alcohols arise by biological reduction from the acids. This assumption was verified when ^{14}C labeled 16:0, 18:1, 18:2 and 18:3 acids were administered as methyl esters to gouramis and high percentages of conversion into the corresponding alcohols were observed.

DONALD M. SAND
HERMANN SCHLENK
University of Minnesota
The Hormel Institute
Austin, Minnesota 55912

ACKNOWLEDGMENT

This investigation was supported in part by grants from the U.S. Public Health Service (HE 05363 and HE 08214) and by The Hormel Foundation. E. C. Ruble and E. W. Vedder gave technical assistance.

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[Received February 12, 1969]

The Relative Turnover of Individual Molecular Species of Phospholipids

The experiments reported here show differences of up to one hundred-fold in the specific radioactivities of individual molecular species of phosphatidyl choline and phosphatidyl ethanolamine of rat liver. Individual molecular species of phospholipids, for example 1-palmitoyl 2-oleoyl phosphatidyl choline, incorporate ^{32}P at rates which are dependent upon the fatty

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18:1	30.1	18.5	51.0	18.6
9,12-18:2	1.3	19.3	13.5	19.6
9,12,15-18:3	0.8	20.6	4.6	20.7
20:0	0.1	(20)	<0.1	(20)
20:1	2.0	20.7	<0.1	20.6
11,14-20:2	1.1	21.6	<0.1	21.5
8,11,14-20:3	<0.1	22.2	0.3	22.1
11,14,17-20:3	0.5	22.6	<0.1	22.5
5, 8,11,14-20:4	<0.1	22.9	1.7	22.8
8,11,14,17-20:4	0.2	23.3	1.0	23.3
5, 8,11,14,17-20:5	0.1	23.9	1.4	23.9
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[a]Incomplete listing.

[b]Equivalent chain length, i.e., retention time in reference to saturated compounds of the same type and chain length, on ethylene glycol succinate at 180 C.

tive identifications. They were repeated on a larger scale to afford samples sufficient for determination of structures. Ozonization and subsequent hydrogenation of ozonides was carried out with the individual compounds or mixtures of isomers (4). The fragments obtained from the alcohol acetates were identified by GLC using authentic aldehyde-acetates as reference compounds. The structures determined by ozonolysis confirmed the tentative identifications of alcohol acetates by GLC. Quantifications were checked by hydrogenation and subsequent GLC. All polyunsaturated structures of alcohols were also found among the acids (Table I). The same identity of structures was encountered with the polyunsaturated alcohols and acids of wax esters from mullet roe.

The structural relationships suggest that the alcohols arise by biological reduction from the acids. This assumption was verified when ^{14}C labeled 16:0, 18:1, 18:2 and 18:3 acids were administered as methyl esters to gouramis and high percentages of conversion into the corresponding alcohols were observed.

DONALD M. SAND
HERMANN SCHLENK
University of Minnesota
The Hormel Institute
Austin, Minnesota 55912

ACKNOWLEDGMENT

This investigation was supported in part by grants from the U.S. Public Health Service (HE 05363 and HE 08214) and by The Hormel Foundation. E. C. Ruble and E. W. Vedder gave technical assistance.

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[Received February 12, 1969]

The Relative Turnover of Individual Molecular Species of Phospholipids

The experiments reported here show differences of up to one hundred-fold in the specific radioactivities of individual molecular species of phosphatidyl choline and phosphatidyl ethanolamine of rat liver. Individual molecular species of phospholipids, for example 1-palmitoyl 2-oleoyl phosphatidyl choline, incorporate ^{32}P at rates which are dependent upon the fatty

TABLE I

Amounts and Relative Specific Radioactivities of Molecules Present in Liver Phosphatidyl Ethanolamines and Phosphatidyl Cholines Following Administration of Radiophosphorus to Rats[a]

Fatty acid at position		Phosphatidyl ethanolamine		Phosphatidyl choline	
1	2	Mole %	Relative specific radioactivity[a]	Mole %	Relative specific radioactivity[a]
16:0	16:1	0.7 ± 0.04		4.6 ± 0.6	6.0 ± 2.0
16:0	18:1	3.0 ± 0.15	54.6	15.5 ± 0.3	
18:0	16:1	0.3 ± 0.02		1.6 ± 0.3	5.0 ± 0.2
18:0	18:1	1.8 ± 0.09		5.5 ± 0.3	0.9 ± 0.4[b]
16:0	18:2	5.7 ± 0.3		6.5 ± 1.3	12.5 ± 1.5
18:0	18:2	3.7 ± 0.3	42.3	11.0 ± 1.0	1.1 ± 0.2
18:1	18:2	2.5 ± 0.2		6.0 ± 1.8	–
16:1	20:4	7.3 ± 2.5	–		
18:0	20:4	32.3 ± 0.6	4.2 ± 0.45	28.3 ± 0.3	0.08 ± 0.02
16:0	22:5	3.8 ± 0.9	5.8 ± 2.2[c]		
18:1	20:4	12.3 ± 0.7		4.3 ± 0.4	0.96 ± 0.03
16:0	20:4	19.6 ± 0.8	9.2 ± 1.7	16.7 ± 0.4	
18:0	22:5	2.8 ± 0.8			
16:0	22:6	1.9 ± 0.15	32.5		
18:0	22:6	1.4 ± 0.13			
18:1	22:6	0.9 ± 0.16			

[a] The relative specific radioactivity is the specific radioactivity expressed as a percentage of the specific radioactivity of the serum inorganic phosphate (96,060 counts/min/μmole). Unless stated otherwise, only those results which differed from zero, as judged by Student's test for P = 0.01, have been reported. Certain components had to be combined in order to obtain statistically significant results.

[b] Significant at P = 0.05 level.

[c] Significant at P = 0.02 level.

acids they contain (1-4). Although a number of workers have calculated turnover times for whole phospholipid classes (5-7), no attempt has been made to derive turnover times for individual molecular species of phospholipids. In order to clarify the metabolic functions of the phosphatidyl cholines, phosphatidyl ethanolamines, etc., it is important to know the amount of each molecular species present and to have some measure of its turnover time. The determination of the turnover times of a group of homologous compounds is difficult (8); nevertheless valuable information regarding the relative turnover times of these compounds can be obtained from the measurement of their specific radioactivities. Keith (9) has shown that the specific radioactivity of inorganic phosphate of rat liver reaches a maximum approximately 70 min after intraperitoneal injection of ^{32}P-orthophosphate. In the present work animals were killed 60 min after ^{32}P injection to ensure that the specific radioactivities of the phospholipid species would be increasing and thus would be measures of their relative turnover times.

Male rats were fed a diet (10) containing 2% of safflower seed oil as the only source of fat. The phospholipids were isolated from the liver and the phosphatidyl cholines and the methyl esters of the dinitrophenylated (MDNP-) phosphatidyl ethanolamines were separated and prepared as described by Collins (4). Both materials were purified by thin layer chromatography (TLC) (9,11) and then subdivided by silver-nitrate TLC (12) to give several fractions differing in their degree of unsaturation. Each of these fractions was examined further by countercurrent distribution (4) using 400 transfers; the amount of phospholipid (as phosphorus (13) or as DNP- groups (14)) and the amount of each fatty acid present in every fifth tube was measured. In the case of phosphatidyl choline the fatty acids at the 1 and 2 positions were distinguished by the use of phospholipase A (4,15); this was not possible for the MDNP-phosphatidyl ethanolamines as the enzyme was without action on these compounds and it was necessary to assume that the polyene fatty acids were exclusively at the 2 position. Fatty acids which were present to the extent of less than 0.6% were ignored. The amounts of molecular species present in each of the fractions subjected to countercurrent distribution were calculated using the procedure described by Collins (16). The amounts (X_{1m},X_{2m},...X_{nm}) of each of the n species present in tube m of one of the countercurrent distributions could then be calculated and the specific radio-

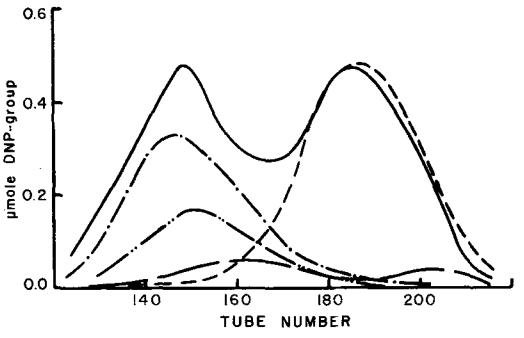

FIG. 1. The countercurrent distribution for 400 transfers of the tetraene fraction of the MDNP-phosphatidyl ethanolamines. The solvent system was heptane-methanol. The fatty acids were determined in every fifth tube: - arachidonic acid, --- palmitic acid, — stearic acid, ---- oleic acid, -- docosapentenoic acid. Whilst the countercurrent distribution of the MDNP-phosphatidyl ethanolamines always gave two peaks, the phosphatidyl cholines gave only one.

activities ($A_1, A_2, \ldots A_n$) of the molecular species were derived from a least-squares fit of a set of simultaneous equations of the type $Y_m = A_1X_{1m} + \ldots A_nX_{nm}$, where the dependent variable Y_m was the amount of radioactivity in the tube $_m$. It is not possible to exclude the possibility that a minor component with a high specific radioactivity may be responsible for the radioactivity of some fractions but the isolation techniques insure that if this is the case the minor component must be chemically and physically very similar.

The phosphatidyl cholines, which yielded a monoene, diene and tetraene fraction were quantitatively isolated (4), separated on the basis of unsaturation by silver nitrate TLC (12), and each fraction was further examined by countercurrent distribution (4). (Table I). Since these specific radioactivities are measures of the relative turnover times of these molecules it can be seen that molecules with palmitic acid in the 1 position always have shorter turnover times than analogous molecules with stearic acid in the 1 position. As predicted by Collins (3), molecules with arachidonic acid have the longest turnover times.

The MDNP-phosphatidyl ethanolamines, which yielded monoene, diene, tetraene and hexaene fractions were examined by the same procedure and the results are shown in Table I. The MDNP-phosphatidyl ethanolamine fractions gave better separations on countercurrent distribution (Fig. 1) than did the phosphatidyl choline fractions; molecules of MDNP-phosphatidyl ethanolamine differing only by two carbon atoms had partition coefficients whose ratio was 1.23 whereas the corresponding figure for the phosphatidyl cholines was 1.07. The amount of MDNP-derivatives obtained (Table I) represented 45% to 50% of the total amino nitrogen in the phospholipids (18). Attempts were made to calculate the specific radioactivities of all molecules present but due to the small amounts of material present in the monoene, diene and hexaene fractions the errors were increased and statistically significant results could only be obtained by combining these fractions as indicated in Table I. The overall incorporation of ^{32}P into the MDNP-phosphatidyl ethanolamines was more than five times that into the phosphatidyl cholines. Again the arachidonoyl species had the longest turnover times. Probably the most striking feature of these results was the high specific radioactivity of the monoene fraction which was greater than 50% of the specific radioactivity of the serum inorganic phosphate. Molecules within this fraction must have very short turnover times.

It is clear that the specific radioactivities and hence turnover times of different phospholipid molecules can vary widely; the ratio of the specific radioactivities of 1-palmitoyl 2-linoleoyl and 1-stearoyl 2-arachidonoyl phosphatidyl choline, for example, was found to be greater than 150:1. De Pury and Collins (17) have postulated that the arachidonoyl phosphatidyl cholines produce more stable membranes because they bind to extracted mitochondria more slowly than do the more saturated phosphatidyl cholines. The finding that the arachidonoyl phosphatidyl cholines have the longest turnover times supports this hypothesis and may indicate that protein-phospholipid binding is an important factor in governing turnover time. Arvidson (19) has separated phosphatidyl cholines and ethanolamines on the basis of unsaturation only and his results for the incorporation of ^{32}P agree with the present results. In addition Arvidson has shown that the incorporation of 1,2 ^{14}C ethanolamine shows a similar heterogeneity in the labelling pattern.

M. A. TREWHELLA
F. D. COLLINS
The Russell Grimwade School of Biochemistry
University of Melbourne
Parkville, Victoria, Australia

ACKNOWLEDGMENT

Supported by the National Health and Medical Research Council, the Australian Research Grants Committee and the A. E. Rowden White Foundation.

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[Received August 27, 1968]

LETTER TO THE EDITOR

Chromatography of Lipids on Polystyrene Gel Column

Sir: Berry and Kaye (Lipids *3*, 386 1968) reported that they were unable to obtain complete separation of polar from non-polar lipids by exclusion chromatography on columns of divinyl cross-linked polystyrene beads by elution with benzene (Tipton, Paulis and Pierson, J. Chromatog. *14*, 486 1964). They concluded that, "at present, polystyrene gel columns are of little or no value for the preliminary separation of polar and non-polar lipids in quantitative analysis". I would like to report that with slight modifications of the method of Tipton et al., although incomplete separation was obtained, 92% of the phospholipid was completely separated from the less polar lipids and the phospholipids appear to be in the native state.

Egg yolk lipid containing 100 mg of phosphorus was freed from water soluble impurities by the method of Wells and Dittmer (Biochemistry *2*, 1259, 1963). It was then applied to a column of polystyrene beads (150 g, Dow Chemical Co., styrene-divinyl benzene copolymer, 2%, 50-100 mesh, lot no. 03055). The polystyrene beads were packed in benzene containing 1% methanol in a column of decreasing diameters (25 x 4, 19 x 3, 19 x 2, 19 x 1.5 cm, made as a single tube) and eluted with the same solvent. The methanol was included in the solvent as an antioxidant. Ten-milliliter fractions were collected and the phospholipid emerged as a symmetrical peak in fractions 17-26. The fractions were examined by infrared spectroscopy and the ratio of the intensities of the bands at 1160 cm^{-1} (C-O stretching of fatty acid esters) and at 1230 cm^{-1} (P=O stretching) used to detect the presence of triglyceride in the phospholipid fractions. Fractions 17-24 were free of triglyceride but there was some overlap in fractions 25-27. Fractions 17-24 were combined and the combined fraction contained 92% of the phospholipid (3.9% phosphorus; Ester/P, 2.0; NH_2/P, 0.25). Similar results have been obtained with chromatograms in which the separation was checked by thin layer chromatography or the determination of ester: phosphorus ratios on each fraction. The use of benzene, rather than 1% methanol in benzene, as an eluent gave similar results. In contrast to Berry and Kaye's results, the percentage of phosphorus in the above sample indicates that there was no contaminating material arising from the polystyrene and eluted from the column with the phospholipid. This difference suggests that there may be some variation in polystyrene samples. The slight overlap of neutral lipid and phospholipid suggests that displacement chromatography (Partridge and Brimley, Biochem. J. *51*, 628 1952) is operating and highlights the obvious advantage of working with successive columns of decreasing diameter.

The results of Collins (Nature, *188*, 297, 1960) and my own observations to be published shortly indicate that considerable chemical change occurs in phospholipid from natural sources during silicic acid chromatography. In contrast polystyrene chromatography represents an extremely mild method for separating neutral lipid from phospholipid and affords 92% recovery of the latter in a native form.

J. B. DAVENPORT
Division of Food Preservation
CSIRO
Ryde, N.S.W. 2112
Australia

[Received November 29, 1968]

The Separation, Structure and Metabolism of Monogalactosyl Diglyceride Species in *Chlorella vulgaris*

B. W. NICHOLS and **R. MOORHOUSE**, Biochemistry Division,
Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford, England

ABSTRACT

The monogalactosyl diglyceride fraction from heterotrophically cultured cells of *Chlorella vulgaris* has been fractionated into species of different fatty acid composition by argentation thin layer chromatography of the unmodified glycolipid. Five major subfractions were isolated, corresponding to groups of molecular species containing 1, 2, 3, 4 and 5 double bonds per molecule, respectively. Incubation of *Chlorella vulgaris* with 2-^{14}C-sodium acetate, followed by radiochemical analysis of the fatty acids of the individual monogalactosyl diglyceride species, demonstrated that at any time the specific activity of a single fatty acid can vary considerably among the various species in which it occurs. These results are consistent with previously reported evidence that significant changes in the fatty acid composition of the monogalactosyl diglyceride fraction of *Chlorella vulgaris* occur following its de novo synthesis.

INTRODUCTION

Although comparisons of the structure and metabolism of individual molecular phospholipid species of different fatty acid content have now been studied in several natural systems, there is little comparable information regarding the galactosyl diglycerides, which quantitatively constitute the major acyl lipids of many plant tissues.

Indeed, little is known even in regard to the general distribution of component fatty acids between the glycerol-1 and -2 positions of the galactosyl diglycerides, apart from the data provided in one recent paper by workers studying these lipids in *Artemisia princeps* leaves (1). However, since some 90% or more of the component fatty acids of the galactosyl diglycerides from these leaves are comprised of a single fatty acid, linolenic acid, the results reported give little indication of what fatty acid distribution might be expected in naturally occurring galactosyl glyceride fractions in which several classes of fatty acid occur in similar proportions.

In previous communications we demonstrated that the fatty acids of the monogalactosyl diglyceride fraction from *Chlorella vulgaris* show a rapid metabolic turnover during photosynthesis, and a direct involvement of this lipid in fatty acid metabolism and synthesis was suggested (2,3). Other metabolic and structural roles have also been proposed for this lipid (4-6), and it is possible that specific species of different fatty acid content may have different functions.

In order to test this possibility, a method for the partial separation of the individual molecular species of the monogalactosyl diglyceride fraction from *Chlorella vulgaris* was developed. This technique was then applied to extracts from cells of *C. vulgaris* which had been incubated with 2-^{14}C-sodium acetate, and the fatty acids of the individual molecular monogalactosyl diglyceride species were then determined quantitatively and radiochemically.

EXPERIMENTAL PROCEDURES

Algae

Chlorella vulgaris, strain no. 211/11h from the Cambridge Collection of Algae and Protozoa, was grown heterotrophically in the light on the rich medium described by Harris et al. (7).

Chlorella cells separated from the culture medium by centrifugation (15 min at 600 g) were twice washed with 0.2 M phosphate buffer, pH 7.4, and were then shaken with a fresh portion of this buffer to give a suspension containing about 12 g wet weight of cells in 250 ml. To this suspension, 60 μC (13.2mg) of 2-^{14}C sodium acetate was added, and the mixture incubated by shaking the flask in the light at 30 C. The light source consisted of four 40 W strip lamps suspended about 25 cm above the incubation flask. Suitable samples were removed from the flask at appropriate intervals.

Extraction and Fractionation of Lipids

Incubations were stopped by pouring the samples into propan-2-ol (20 vol). The resultant mixture was then shaken and filtered and the residue re-extracted with chloroform-methanol (2:1 v/v). The combined filtrates were concen-

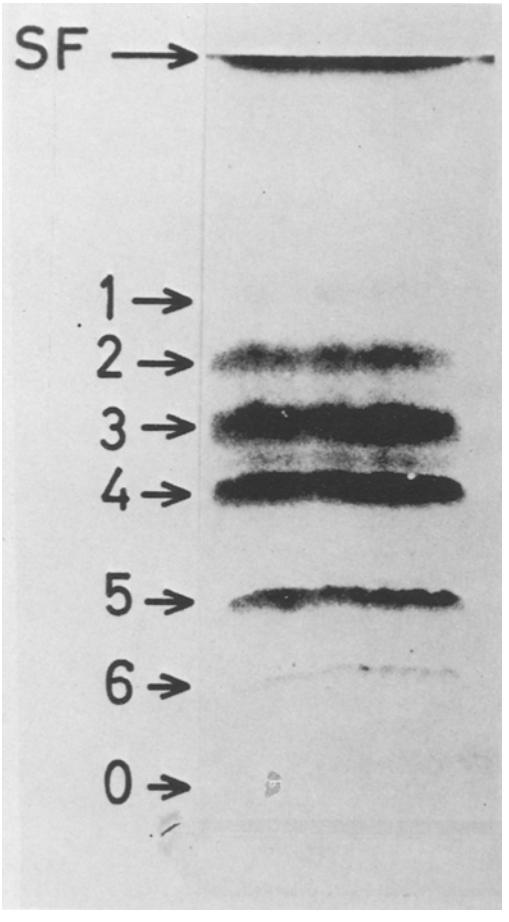

FIG. 1. Argentation-TLC of a monogalactosyl diglyceride fraction from *Chlorella vulgaris*. The solvent was chloroform-methanol-water (60:21:4 v/v). O, origin; SF, solvent front; 1, 2, 3, 4, 5, fractions containing 1, 2, 3, 4 and 5 double bonds per molecule, respectively; 6, fraction probably containing 6 double bonds per molecule. The fractions were located by spraying the chromatogram with chlorsulfonic acid, followed by heating to 230 C for 10 min.

trated in vacuo and washed with 0.9% NaCl to remove water-soluble impurities.

The lipid extracts from each incubation were partially fractionated by column chromatography on DEAE cellulose (acetate form) (8). The resultant monogalactosyl diglyceride fractions were invariably contaminated with pigment, and were purified from this material by preparative thin layer chromatography (TLC) on 0.5 mm layers of silica gel employing chloroform-methanol-acetic acid-water (200:20:5:2 v/v) as mobile phase.

Separation of Monogalactosyl Diglyceride Species

Monogalactosyl diglyceride was fractionated into species of differing fatty acid content by argentation-TLC on 0.25 mm layers of silica gel (without gypsum binder) containing 10% silver nitrate. The chromatoplates employed were dried for 1 hr at 100 C immediately after preparation, and were then stored in a darkened glass tank containing saturated calcium chloride solution. Chloroform-methanol-water (60:21:4 v/v) gave resolutions superior to those obtained with other solvent systems tried as mobile phases.

When used for preparative purposes, the mobile phase contained 0.005% butylated hydroxy toluene (BHT) to prevent oxidation of lipids during detection and removal from the chromatograms. Detection of individual species was achieved by spraying the chromatograms with a 0.05% methanolic solution of dichlorofluorescein, followed by visualization under uv light. The relevant bands were then scraped from the chromatogram and the lipid fractions eluted from the adsorbent with chloroform-methanol (2:1 v/v, then 1:2 v/v). Silver nitrate was removed by washing the extracts with water.

The purity of each fraction was checked by TLC on silica gel and on silver nitrate-impregnated silica gel.

Identity and Radioactivity of Fatty Acids

Lipids were refluxed with methanol-benzene-concentrated H_2SO_4 (20:10:1 v/v) for

TABLE I

Fatty Acid Composition of Individual Species of Monogalactosyl Diglyceride Isolated From Heterotrophically Cultured Cells of *Chlorella vulgaris* After Incubation in the Light for 24 hr

	Fatty Acid											
Fraction	12:0	14:0	14:1	14:2	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3
Total	t	1	1	t	3	12	22	2	1	17	29	11
1	0	9	t	0	28	6	0	0	4	50	3	0
2	0	t	t	t	2	39	t	0	0	52	7	0
3	0	0	t	t	t	23	18	0	0	26	34	0
4	0	0	0	0	t	2	41	0	0	3	53	0
5	0	0	0	0	2	2	35	3	t	3	14	40

90 min and the reaction mixture then diluted with water. The methyl esters of the component fatty acids were extracted with light petroleum (bp 60-80 C), dried, and analyzed on a radiochemical gas chromatograph employing diethylene glycol adipate as stationary phase. Comparative specific activities are expressed as ratios of radioactivity peak area to mass peak area.

When radiochemical analyses were not required, methyl esters were analyzed on a Pye 104 gas chromatograph connected to a flame ionisation detector, using either polyethylene glycol adipate or silicone SE-30 (Applied Science Laboratories, State College, Pa.) as stationary phase.

RESULTS

Figure 1 illustrates the separations obtained by argentation-TLC on the monogalactosyl diglyceride fraction from heterotrophically cultured *Chlorella vulgaris*, which was resolved into five major species. Two minor fractions, one running between fractions 3 and 4 and the other situated between fraction 5 and the origin (Fig. 1), were not isolated in sufficient quantities to permit a complete characterization of the identity and radioactivity of their component fatty acids.

Dichlorofluorescein is a more sensitive detection reagent than chlorsulfonic acid in this system, and consequently a more clearcut resolution than that depicted in Figure 1 is experimentally possible.

Analysis of the component fatty acids of the major monogalactosyl diglyceride fractions (Fig. 2, Table I) shows that they correspond to molecular species, or groups of molecular species, containing 1, 2, 3, 4 and 5 double bonds per molecule respectively.

Little change in fatty acid composition of the total monogalactosyl diglyceride fraction occurred during the incubation period, so that figures for the 24 hr incubation only are given.

The specific activities of the fatty acids within these species are illustrated and recorded in Table II and Figure 3.

DISCUSSION

While the separation of neutral lipid classes (e.g., diglycerides and triglycerides) into molecular species of differing fatty acid content is comparatively easily achieved by column or thin layer chromatography on adsorbents impregnated with silver nitrate (9), the equivalent resolution of more polar lipids is generally less easy.

Thus while Arvidson has reported fractionation of animal lecithins into molecular species using argentation-TLC (10) and argentation-reverse phase-TLC (11), other authors have preferred to modify these lipids by removal or modification of the more polar groups before attempting fractionation (12-15).

The results obtained in the present work demonstrate that good separations of monogalactosyl diglyceride species can be obtained by argentation-TLC of the unmodified glycolipid.

The outstanding feature of the fatty acid composition of each monogalactosyl diglyceride fraction is that in every case the major component acids do not differ in degree of unsaturation by more than one double bond (Table I). Thus the fraction containing an overall 2 double bonds per glycolipid molecule is almost entirely composed of species containing 2 monoenoic acids per molecule. Only small proportions of species containing saturated and dienoic acids are present. Similarly the fractions containing 3 and 4 double bonds per molecule contain no detectable quantities of molecules containing trienoic acids associated with saturated and monoenoic acids respectively. The only fraction which contained trienoic acids was that which possessed mainly octadecatrienoic and hexadecatrienoic acids in conjunction with octadecadienoic and hexadecadienoic acids.

The presence of small quantities of saturated and (labeled) monounsaturated fatty acids in the fractions containing predominantly 4 and 5 double bonds per molecule cannot be explained at present. To account for their presence in fractions of such polarity they would have to occur in molecules also containing equivalent quantities trienoic and tetraenoic acids, respectively, and these were not detected in our preparations.

Monogalactosyl diglyceride species containing 6 double bonds per molecule do not occur in significant proportions in heterotrophically cultured *Chlorella vulgaris*, but when this alga is grown photoautotrophically the overall level of trienoic acids in the cell is considerably enhanced (16) and in these circumstances glycolipid species containing 6 double bonds per molecule occur in appreciable amounts.

These results indicate that the distribution of individual fatty acids within the monogalactosyl diglyceride fraction of *Chlorella vulgaris* differs from that established for other polar lipids, particularly phospholipids. In the case of the latter classes of naturally occurring lipid, saturated acids tend to predominate at the

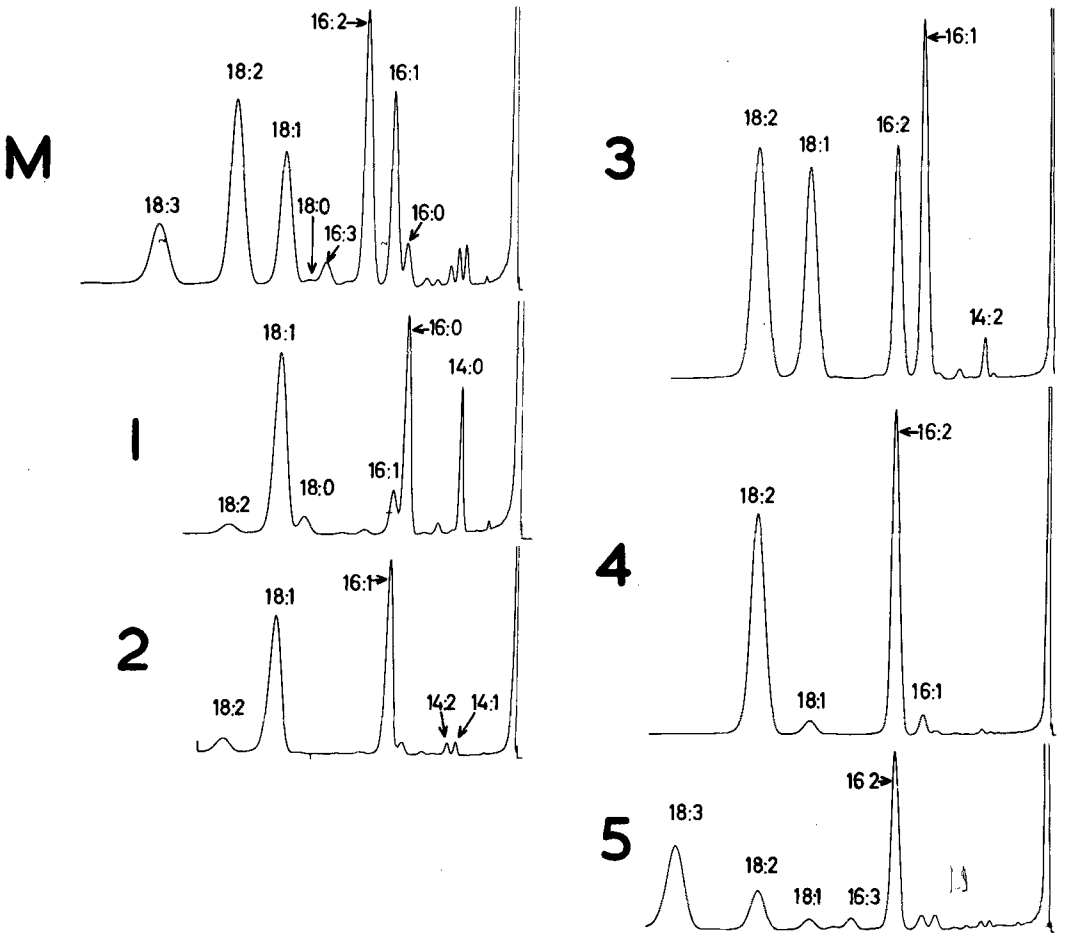

FIG. 2. GLC fractionation of the methyl esters of the component fatty acids of monogalactosyl diglyceride species. The stationary phase was diethylene glycol adipate. M, unfractionated monogalactosyl diglyceride; 1, 2, 3, 4 and 5, species isolated by argentation-TLC.

TABLE II

Relative Specific Activities of Component Acids of Monogalactosyl Diglyceride Species After Incubation of *Chlorella* With 2-^{14}C Acetate for 2 hr and 24 hr Periods

	Fatty Acid										
Fraction	14:0	14:1	14:2	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3
After 2 hr											
Total	120	50		40	118	3	0	100	135	4	0
1	670	500		80	300			80	320		
2		40			195				184		
3					25	11			30	12	
4					0	0			0	0	
5					0	0	0		0	0	0
After 24 hr											
Total	320	60	50	300	200	30	t	400	200	20	3
1	300			368	455			525	322		
2		100	40		237				237	t	
3		14			53	34			74	29	
4					63	14			40	13	
5				0	73	6	t	0	125	9	4

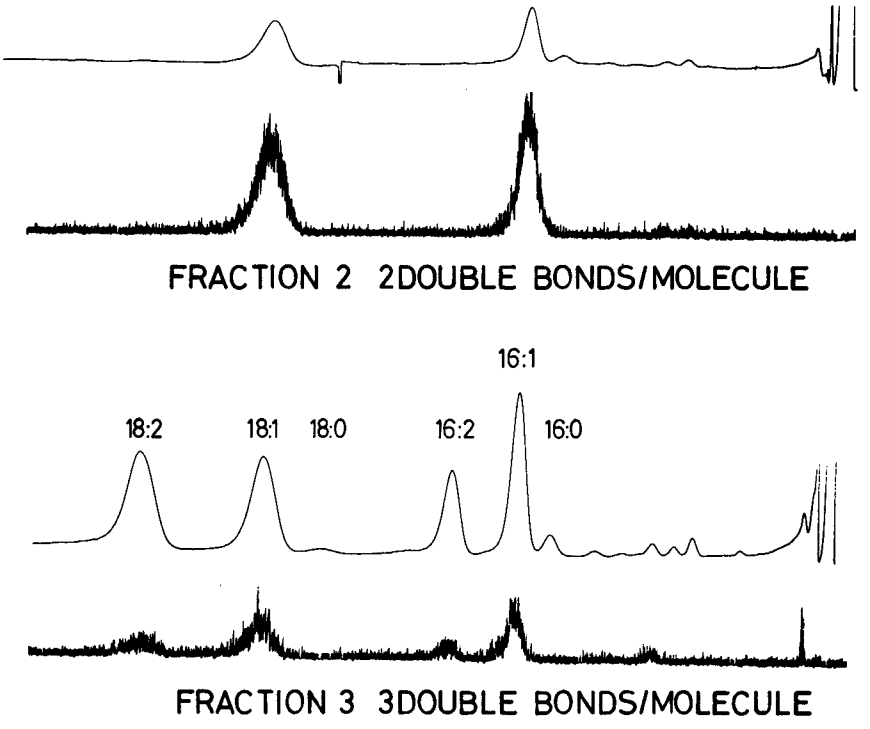

FIG. 3. Radio-gas chromatogram of methyl esters from species 2 and 3 after incubation of *Chlorella* with ^{14}C acetate for 2 hr. Upper chromatogram of each pair is the mass trace; the lower trace records ^{14}C activity.

glycerol 1 position and the more highly unsaturated acids at the 2 position (17,18). Consequently most naturally occurring phospholipid fractions contain a large proportion of species in which saturated acids occur in the same molecule as highly unsaturated acids (10,12,13,14,17,19).

Our present inability to achieve controlled partial deacylation of monogalactosyl diglyceride (to monogalactosyl monoglyceride and free fatty acid) has so far prevented a complete determination of the structure of the individual molecular species of this glycolipid, but the results described in this paper clearly indicate that such pronounced differences in degree of unsaturation between acids attached at the 1 and 2 positions as occur in natural phospholipids do not occur in the monogalactosyl diglyceride fraction from *Chlorella*.

These differences could either reflect differences in the specificities of the acyl transferases involved in synthesizing the diglyceride precursors of these classes of compound, or derive from changes in fatty structure which may occur in the lipids following their de novo synthesis. The latter scheme is consistent with previously reported data relating to monogalactosyl diglyceride metabolism in *Chlorella vulgaris* (2,3).

Study of the data presented in Figure 3 and Table II reveals that at any one time the specific activity of an individual fatty acid varied considerably among the different species. For example, after 2 hr incubation the specific activities of the monoenoic acids in species containing two double bonds are more than six times greater than those of the same acids in species containing three double bonds per molecule (Fig. 3, Table II).

Variations of this nature would be expected if certain species were more active metabolically than others, since the former might be expected to be synthesized at the faster rate. Other workers have shown such differences in the rates of synthesis of individual phospholipid species in animal tissues (17,20-22).

Alternatively, the reported data may also be explained by the occurrence of a series of alterations of fatty acid structure taking place within the monogalactosyl diglyceride molecule subsequent to de novo synthesis from saturated acids. During the course of the incubation these acids may then be systematically desaturated according to the established pathway (23), and

the label would consequently pass steadily from species of a lower degree of unsaturation to those more highly unsaturated. Consideration of the data in Table II shows that they are generally consistent with such a system. Thus after 2 hr the only dienoic acids labeled are those in the species containing three double bonds per molecule (Table II). After 24 hr, however, a large proportion of the labeled dienoates are found in the species containing four and five double bonds per molecule (Table II).

Clearly the experiments so far performed do not conclusively decide which of these alternative mechanisms is operative, although the second seems more likely, and it is possible that both systems make some contribution to the overall metabolism of monogalactosyl diglyceride in *Chlorella*.

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[Received November 5, 1968]

Dihydrosterculic Acid, a Major Fatty Acid Component of *Euphoria longana* Seed Oil[1]

R. KLEIMAN, F. R. EARLE and I. A. WOLFF,
Northern Regional Research Laboratory[2], Peoria, Illinois 61604

ABSTRACT

The seed oil of *Euphoria longana*, Sapindaceae, contains 17.4% of 9,10-methyleneoctadecanoic (dihydrosterculic) acid. This identification is based on information from thin layer chromatography, infrared analysis, gas liquid chromatography, nuclear magnetic resonance and mass spectroscopy. Since GLC of the oil showed components that emerged between the usual triglycerides, the cyclopropanoid acid is apparently a triglyceride constituent. The presence of smaller amounts, less than 1%, of cyclopropanoid fatty acids of different chain lengths is indicated by GLC and TLC analyses of the methyl esters. The other major fatty acids in this oil are: 16:0 (19%), 18:0 (7%), 18:1 (36%), 18:2 (6%), 18:3 (5%) and 20:0 (4%). *Euphoria* oil contains considerably larger amounts of cyclopropanoid fatty acids than previously reported in other seed oils.

INTRODUCTION

Seed of *Euphoria longana* Lam., a member of the Sapindaceae, was examined during continuing investigations of the chemical composition of plants. *E. longana* is a tree, 10-14 m high, grown in the warm regions of India for its edible fruit (Barclay, private communication). This species has little potential as a commercial oil source (4% oil in seed), but its oil is of interest because it contains 17% of a major component now identified as *cis*-9,10-methyleneoctadecanoic (dihydrosterculic) acid. This concentration of cyclopropanoid fatty acid is the highest so far reported in any seed oil.

Cyclopropanoid fatty acids have been identified in microorganisms (1) and in seed oils of plants in the order Malvales (2). In seed oils, cyclopropanoid fatty acids were previously found in small amounts, 1-2%, accompanied by larger amounts of cyclopropenoid fatty acids. No cyclopropenoid fatty acids were observed in *E. longana* seed oil.

EXPERIMENTAL PROCEDURES

Seed collection, cleaning, oil extraction and oil analysis were conducted as previously described (3).

Methyl esters were prepared from the extracted oil with BF_3-methanol reagent by the method of Metcalfe et al. (4).

Esters were analyzed by gas liquid chromatography (GLC) as before (5) or with an F&M model 402 instrument equipped with a 12 ft x ¼ in. column packed with 5% LAC-2-R 446 and a 4 ft x ¼ in. column packed with 5% Apiezon L, both at 200 C.

GLC of the whole oil was carried out with an F&M model 5750 instrument in accordance with the procedure of Litchfield et al. (6).

Methyl dihydrosterculate was isolated by preparative GLC with an Autoprep model A-700 instrument equipped with a 10 ft x ¼ in. aluminum column packed with 20% Apiezon L and operated at 230 C.

Infrared (IR) analyses were performed on either a Perkin-Elmer IR-137 or IR-337 spectrophotometer. Samples were analyzed either as films on NaCl disks or as 1% solutions in 1 mm NaCl cells with CCl_4 or CS_2 as the solvent.

Thin layer chromatography (TLC) was carried out on 20 x 20 cm plates spread with a 275 μ layer of Silica Gel G impregnated with 20% $AgNO_3$. Preparative TLC was done with 10 x 36 cm plates spread with a 1 mm layer of Silica Gel G, also impregnated with 20% $AgNO_3$. Benzene was the developing solvent for both.

A Varian HA-100 spectrometer was used to measure nuclear magnetic resonance (NMR) in deuteriochloroform solution with and without tetramethylsilane as the internal standard.

Double bond location was determined by reductive ozonolysis and subsequent GLC of the resulting fragments (7,8).

Methyl dihydrosterculate was reduced with hydrogen on Adam's catalyst, and the products were analyzed by mass spectroscopy with an LKB gas chromatograph-mass spectrometer at 20 eV (9).

RESULTS AND DISCUSSION

GLC analysis of the methyl esters of *E. longana* seed oil (Table I) revealed a component (17%) with equivalent chain lengths (ECLs) of

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[1]Presented at the AOCS-AACC Joint Meeting, Washington, D.C., April 1968.

[2]No. Utiliz. Res. Dev. Div.; ARS, USDA.

TABLE I

Composition of *Euphoria longana* Seed Oil

Ester	Per cent	ECL of cyclopropanoid esters	
		Apiezon L	LAC-2-R 446
14:0	0.3		
C_{15} cyclopropane	0.4	14.8	15.3
16:0	19		
17:0	Trace		
C_{17} cyclopropane	0.7	16.8	17.4
18:0	7		
18:1	36		
18:2	6		
18:3	5		
C_{19} cyclopropane	17	18.8	19.4
20:0	4		
20:1	0.9		
22:0	3		
24:0	1		

19.4 (from LAC-2-R 446 column) and 18.8 (from Apiezon L column). From these ECLs it was first postulated that this component was a C_{19} monoenoic ester since each ECL is one unit longer than that of methyl oleate. GLC of the oil itself (Fig. 1) seemed to substantiate this postulation since glycerides with odd carbon numbers were observed. This analysis also indicated that the unusual acyl group is a triglyceride constituent.

Isolation of *cis*-monoenoic esters by preparative TLC with Silica Gel G impregnated with $AgNO_3$ yielded only 18:1. GLC of the saturated esters from the TLC separation, however, showed the presence of 34% of one unusual component and smaller amounts of esters with ECLs that identified them as homologues, with two and four fewer carbon atoms, of this component (Table I).

The GLC and TLC characteristics of the unusual ester were not altered during preparative GLC.

The acid, recovered from saponification of the ester, exhibited the IR 9.8 μ band, which is

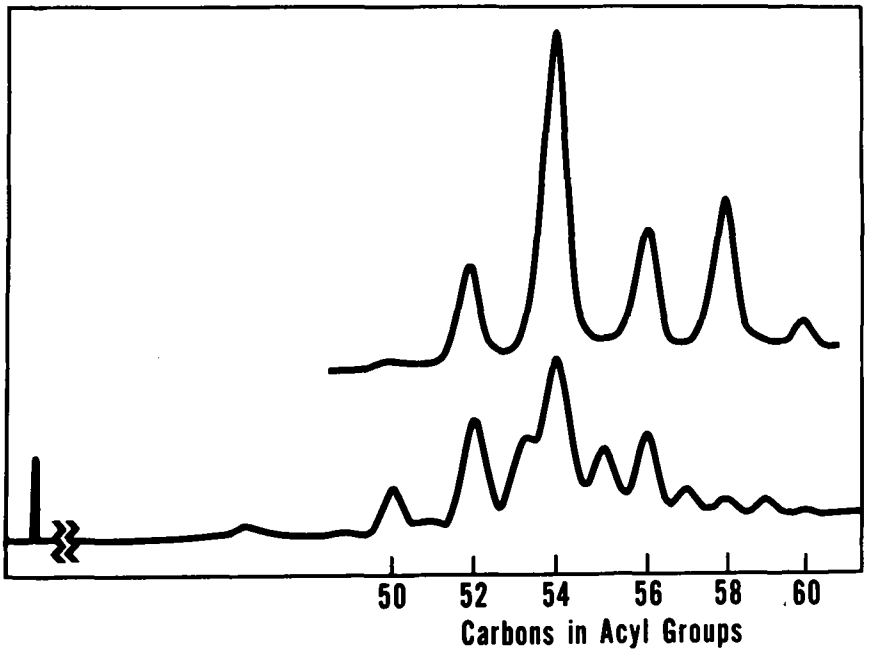

FIG. 1. GLC of *Euphoria longana* oil (bottom curve). Top curve represents an oil with acyl groups containing only even numbers of carbon atoms and is shown for comparison.

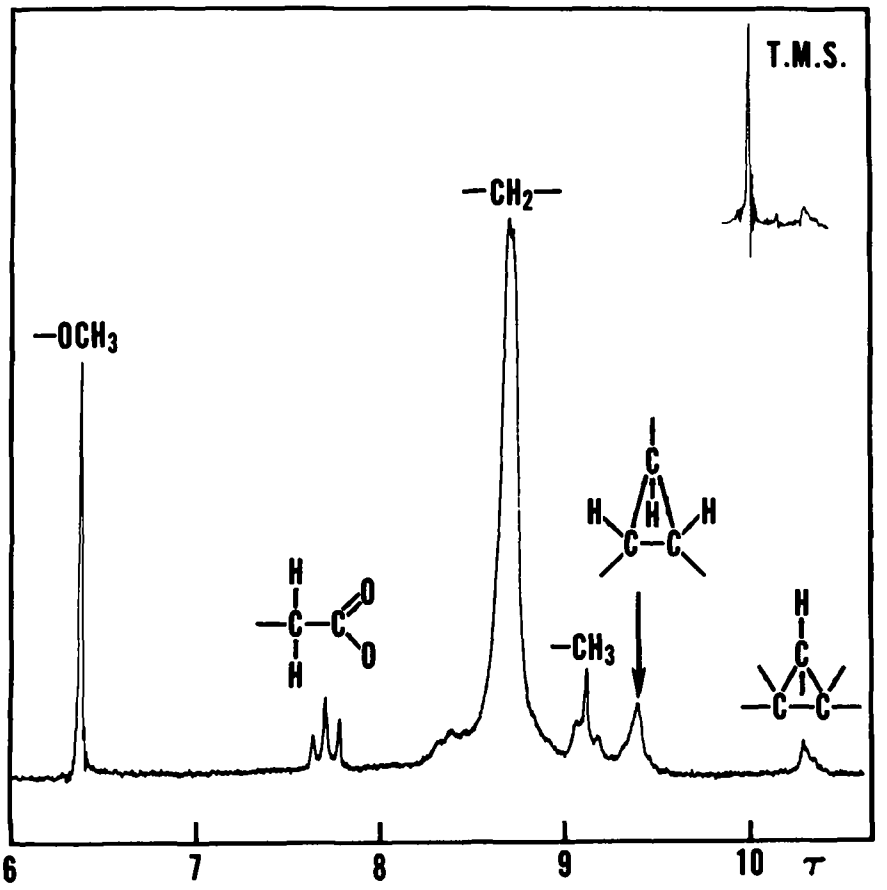

FIG. 2. NMR spectrum of methyl dihydrosterculate from *E. longana* seed oil.

indicative of a cyclopropane ring (10). The rest of the spectrum was the same as that of stearic acid.

NMR analysis of the isolated ester (Fig. 2) also indicated the presence of the cyclopropanoid functional group. The bands at 9.4 and 10.3 τ (ratio of 3:1) showed the presence of a *cis*-cyclopropane ring (11). The spectrum was consistent with that expected from a saturated methyl ester, in both band location and quantitation.

Cis configuration for the cyclopropanoid ester was also suggested by its GLC mobility (ECLs) which agreed closely on both polar and nonpolar phases with ECLs reported (12) for a C_{19} compound with the *cis* ring function at C-9,10. The isomeric *trans*-cyclopropanoid ester had significantly lower ECL values (12).

Cyclopropanoid fatty esters undergo hydrogenolysis (1); the products from dihydrosterculate are the 9-methyl and 10-methyl C_{18} branched-chain esters and the C_{19} straight-chain ester. This reaction was used by McCloskey and Law (9) to produce from cyclopropanoid fatty esters derivatives that are useful in locating the ring by mass spectrometric analysis.

The mass spectrum of the methyl-branched esters derived from the cyclopropanoid ester from *E. longana* was identical to that of the methyl-branched esters from known methyl dihydrosterculate. The structure determining peaks (m/e = 157, 158, 159, 171, 172, 173, 153, 167, 185, 199, 135 and 149) (9) are uniquely derived from methyl dihydrosterculate among the hydrogenated C_{19} cyclopropanoid esters.

The unusual acid found from *E. longana* is thus established as *cis*-9,10-methyleneoctadecanoic (dihydrosterculic) acid.

The major monoenoic acid occurring with the dihydrosterculic is oleic; reductive ozonolysis of the monoenoic ester and subsequent GLC results in only two peaks, nonanal and methyl azela-aldehydate. The absence of *trans* (10.36 μ) absorption in the IR

of the whole oil, together with results from the ozonolysis, confirms that the monoenoic ester is essentially all methyl oleate.

The absence of any significant number of acyl groups with the cyclopropene function in *E. longana* oil was demonstrated by a negative Halphen test (13) and nonreactivity to HBr (13). [In this reference, *E. longana* was listed under the synonym *Dimocarpus longan* (Item 891) and erroneously placed among the Euphorbiaceae]

The complete ester composition of *E. longana* is given in Table I. The unusual features of the composition are the large amount of dihydrosterculate and the presence of small amounts of shorter chain esters, which are probably cyclopropanoid. Long-chain esters (C_{20-22}) are also present but in smaller amounts than previously reported in other members of the Sapindaceae family (14).

ACKNOWLEDGMENTS

J. A. McCloskey, Baylor University College of Medicine, for mass spectral data; W. A. Boyd for NMR; and U.S. Plant Introduction Station, Miami, Florida, for seeds.

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[Received December 6, 1968]

The Role of Lipids in Heme Synthesis

YOSHIMASA YONEYAMA, HIDEO SAWADA, MASAZUMI TAKESHITA and **YOSHIKI SUGITA,**
Department of Biochemistry, School of Medicine, University of Kanazawa, Kanazawa, Ishikawa, Japan

ABSTRACT

The effect of lipids on protoheme ferrolyase, which combines ferrous iron with protoporphyrin, was investigated. The enzyme extracted with 1% Na cholate from acetone-dried powder of chicken erythrocyte stroma showed high enzymic activity, while that extracted with 0.4 M KCl showed little activity. The former contained lipids, the main components of which were lecithin and phosphatidylethanolamine, whereas the latter contained little lipid. Crude lipids of several sources restored the enzyme activity of 0.4 M KCl extracts. The activating effects of purified lipids, especially phospholipids which showed the strongest activation, were further examined. Phospholipids were divided into three groups: the choline-containing group, lecithin and sphingomyelin, was inhibitory or slightly accelerative on heme synthesis; the acidic phospholipids, namely phosphatidylethanolamine, cardiolipin, phosphatidic acid and phosphatidylinositol, were strong activators and the intensity of activation was in the order of the acidity of the phospholipids; and the lysophospholipids, namely lysolecithin, lysophosphatidylethanolamine and sphingosylphosphorylcholine, activated the heme synthesis most effectively. In the presence of cholate, choline-containing lipids were highly effective, while acidic phospholipids were inhibitory and lysophospholipids were neutral. Palmitic acid showed slight stimulative effect. Tripalmitin was neutral or inhibitory. Anionic, cationic and neutral synthetic detergents were slightly stimulative in low concentration and inhibitory in high concentration. An activation mechanism of phospholipids was proposed in which the hydrophilic anionic part of lipid in the lipoprotein enzyme molecule attracts ferrous iron. After being stripped of solvation water, the ferrous iron is transferred to the hydrophobic part of the enzyme molecule to be inserted into porphyrin.

INTRODUCTION

The combination of iron and protoporphyrin is an important enzymic reaction for living organisms as the last step of heme formation, and has been investigated particularly as a part of hemoglobin synthesis in higher animals. The enzyme, protoheme ferrolyase (EC 4.99,1.1) is a particulate, mitochondrial enzyme. The authors have reported the extraction of the enzyme from duck erythrocyte stroma (1) or rat liver mitochondria (2) and have described some of its properties. The enzyme could be solubilized only with the aid of detergents such as cholate or Tweens. Every effort to purify the enzyme without detergents has been unsuccessful. In previous communications (3,4), the authors suggested the participation of lipid in duck erythrocyte enzyme. The present paper deals with further study on the role of lipids in protoheme ferrolyase from chicken erythrocyte stroma with special attention to the effects of various purified phospholipids.

MATERIALS AND METHODS

Materials

Protoporphyrin was prepared from protohemin by the method of Grinstein (5). Lecithin was prepared from egg yolk according to Pangborn (6) and purified further by silicic acid chromatography. Phosphatidylethanolamine was obtained from egg yolk according to Lea et al. (7). Phosphatidic acid was obtained from egg yolk lecithin by treatment with phospholipase D from cabbage and purified according to Kates (8). Lysolecithin was obtained from egg yolk lecithin after Naja naja venom treatment and purified by silicic acid chromatography. Lysophosphatidylethanolamine was prepared from egg yolk lipid according to Matsumoto (9) with slight modifications. Phosphatidylmonoinositol from soy bean, cardiolipin from ox heart, sphingomyelin from human erythrocyte and sphingosylphosphorylcholine (10) were gifts of Kikkoman Co., Ltd., Sumitomo Chemical Industry Co. Ltd., Prof. Yamakawa and Taketomi, respectively. The purity of lipids was checked by thin layer chromatography. Naja naja venom was a product of Sigma.

Preparation and Assay of the Enzyme

The enzyme was prepared from the acetone powder of chicken erythrocyte stroma according to the method previously reported for duck erythrocyte stroma (1). The acetone-dried powder of chicken erythrocyte stroma

TABLE I

Comparison of Enzyme Preparations
Extracted With Sodium Cholate and 0.4M KCl

Medium	Protein mg/ml	Phospholipids		Activity μmoles/mg protein
		Lipid P μmoles/ml	PLC/PLE	
0.1M KCL + 1% Sod. cholate	2.8	2.07	1.6	5.38
0.4M KCl	2.0	0.01		0.18

was extracted either with a solution containing 0.05 M Tris buffer (pH 8.0), 0.1 M KCl and 1% Na cholate or a solution containing 0.05 M Tris buffer (pH 8.0) and 0.4 M KCl for 15 hr at 4 C. The extract was centrifuged for 15 min at 10,000 *g* and the supernatant was used as the enzyme. The incubation mixture contained 0.1 μmole of $FeCl_3.6H_2O$ labeled with 4 - 5 x 10^5 cpm of $^{59}FeCl_3$, 0.1 μmole of protoporphyrin, 10 μmoles of cysteine, 100 μmoles of 2-mercaptoethanol, 150 μmoles of Tris buffer at pH 8.3, enzyme and lipid in a total volume of 2.0 ml. Incubation was carried out anaerobically in a Thunberg tube at 37 C for 30 min. After incubation, carrier hemoglobin was added and ^{59}Fe-hemin was crystallized, counted and estimated as previously reported (1).

RESULTS

Comparison of Cholate Extract and Extract With 0.4 M KCl

Sodium cholate extract contained lipids in addition to the protein with high enzyme activity, whereas 0.4 M KCl extract contained only the protein with little enzyme activity and no lipid, as shown in Table I. Acetone powder of chicken erythrocite stroma was extracted with 0.1 M KCl - 1% sodium cholate or with 0.4 M KCl. The supernatants were analyzed and assayed. Lipids extracted with sodium cholate were analyzed by thin layer chromatography.

TABLE II

Effect of Lipid of Acetone Powder
of Chicken Erythrocyte Stroma on Enzyme Activity

Tube number	Lipid, mg	Na cholate, mg	Hemin formed, μmoles/mg protein
1	0	0	0.45
2	0.83	0	1.72
3	1.66	0	4.71
4	0	5.0	3.15
5	0.83	5.0	7.58
6	1.66	5.0	6.61

Main components were lecithin and phosphatidylethanolamine; sphingomyelin was also detected.

Effects of Crude Lipids on Enzyme Activity

Since the effects of lipids on the enzyme activity were suggested, crude lipids from chicken erythrocyte stroma and from egg yolk were tested for their activity in the enzymatic combination of iron and protoporphyrin. The effects of crude lipids, extracted with chloroform-methanol (1:1) from acetone-dried powder of chicken erythrocyte stroma, on the 0.4 M KCl extract are shown on Table II. Lipid and sodium cholate were added to the standard incubation mixture. Stimulating effects were observed in the absence of cholate, and weak acceleration in the presence of cholate.

Lipids extracted directly with chloroform-methanol and those extracted first with cholate and then chloroform-methanol were almost the same in thin layer chromatography. The crude lipid extracted with acetone from egg yolk was composed mainly of lecithin and simple lipids. Its effects on the enzyme activity were almost the same as those obtained from acetone-dried powder of chicken erythrocyte stroma. The lipids extracted with ethanol from acetone-treated egg yolk cake and their degradation products by Naja naja venom were analyzed with thin layer chromatography. The chief component of the lipids before the venom treatment were lecithin and phosphatidylethanolamine. After the treatment lecithin was decreased, phosphatidylethanolamine disappeared, lysolecithin and lysophosphatidylethanolamine were increased and fatty acid appeared (Fig. 1). The effects of egg yolk lipids before and after the venom digestion are shown in Table III. Aqueous emulsion of ethanol extracted lipid of egg yolk cake was digested with Naja naja venom. The digested liquid was successively transferred to ethanol, *n*-hexane and ethanol layers. From the ethanol solution aqueons emulsion of lipid was obtained and added to the standard incubation mixture.

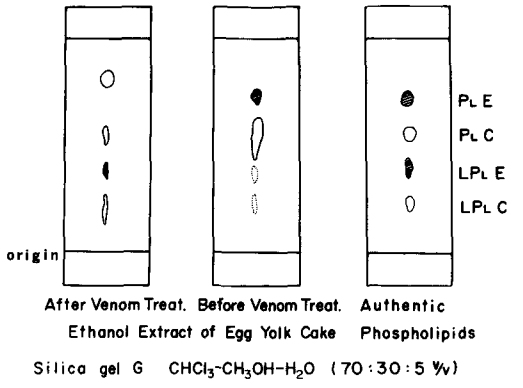

FIG. 1. Thin layer chromatograms of egg yolk lipid before and after venom digestion. Shadowed areas are ninhydrin positive.

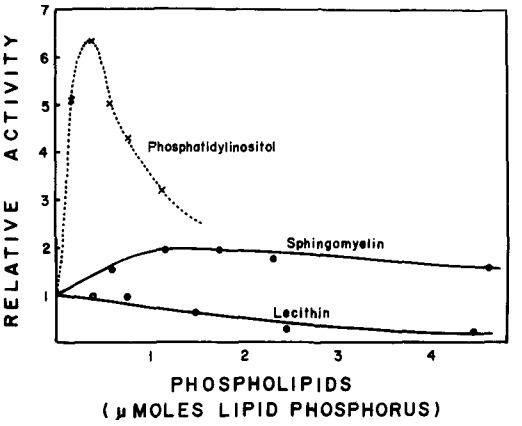

FIG. 2. Relative activity of choline-containing phospholipids.

Before digestion, the results were similar to those of acetone-extracted egg yolk lipids and of chicken erythrocyte lipids, but after digestion, accelerating effect was increased in the absence of cholate, whereas in its presence the effect was decreased. This change in activating effect after phospholipase treatment suggested that the effect was due to the presence of phospholipids

Effects of Purified Lipids on Enzyme Activity

Phospholipids. Phospholipids tested were divided into three groups according to their action on the 0.4 M KCl extracted enzyme, choline containing, acidic and lyso-phospholipids.

Choline containing phospholipids, lecithin and sphingomyelin were inhibitory or slightly accelerative on the enzyme activity as shown in Figure 2. The activity without lipids is taken as a standard and others are given as relative values. Phosphatidylinositol, an acidic phospholipid, is included for comparison. The effects of acidic phospholipids, namely phosphatidylethanolamine, cardiolipin, phosphatidic acid and phosphatidylinositol are shown in Figure 3. Lecithin, a choline containing phospholipid, is included for comparison. They activated heme formation strongly and the intensity of activation was in the order of the acidity of the phospholipids. Lysophospholipids were the most effective group of the phospholipids. The effects of lysolecithin, lysophosphatidylethanolamine and sphingosylphosphorylcholine (lysosphingomyelin) are shown in Figure 4, together with their original lipids. At low concentration lysolecithin was the most effective stimulator, and at high concentration lysophosphatidylethanolamine was the strongest. It is interesting that lysolecithin showed a strong acceleration on enzyme activity whereas its original lipid, lecithin was inhibitory.

In the presence of sodium cholate, which was a moderate accelerator by itself, the effects of phospholipids were different from those in the absence of cholate. The effects of three groups of phospholipids in the absence of cholate are shown in Figure 5. Lecithin and sphingomyelin, which showed little activation in the absence of cholate, activated the heme

TABLE III

Effect of Pretreatment of Egg Yolk Lipid With Phospholipase on Enzyme Activity

Tube number	Lipid before treatment, mg	Lipid after treatment, mg	Na, cholate, mg	Hemin formed, μmoles/mg protein
1	0	0	0	0.20
2	1.10	0	0	0.27
3	0	1.26	0	1.25
4	0	0	5.0	0.65
5	0.55	0	5.0	3.14
6	1.10	0	5.0	3.05
7	0	0.63	5.0	1.11
8	0	1.26	5.0	0.98

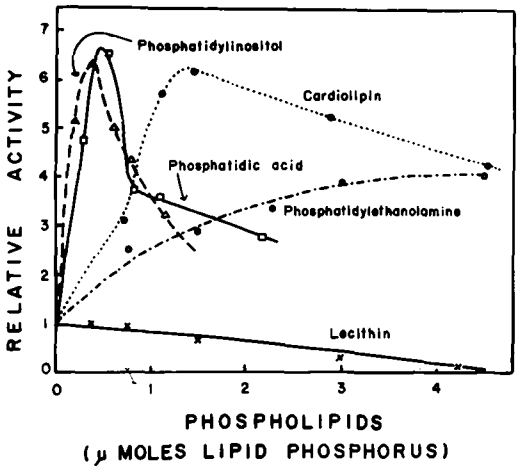

FIG. 3. Relative activity of acidic phospholipids.

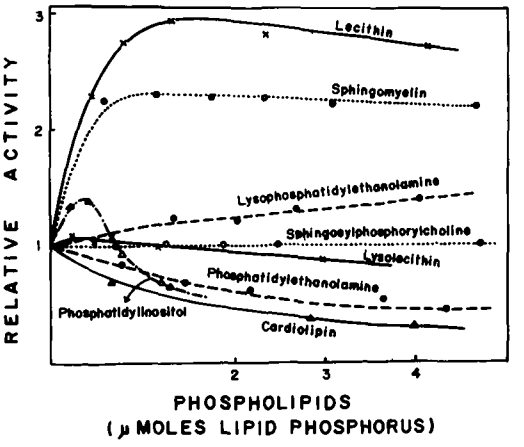

FIG. 5. Relative activity of phospholipids in the presence of cholate.

formation. Acidic phospholipids acted as inhibitors in the presence of cholate. Lysophospholipids, which were the most effective activators in the absence of cholate, were neutral in the presence of cholate.

The data for lecithin in the absence and presence of sodium cholate, expressed as μmoles hemin synthesized, are given in Figure 6.

Simple Lipids. The effects of palmitic acid on the enzyme are shown in Figure 7. The weak stimulative effect of palmitic acid was very small compared with stimulation by a phospholipid such as lysolecithin. Tripalmitin was neutral on the enzyme and was inhibitory in the presence of cholate.

Effects of Synthetic Detergents on Enzyme Activity

Sodium dodecylsulfate, cetylpyridinum bromide and Tween 20 showed slight stimulative effect on enzyme activity in a very low concentration range. At higher concentrations they were inhibitory. The results with sodium dodecylsulfate are given in Figure 8. Slight accelerative effects, observed in those detergents irrespective of their charges, may be due to their general surface active properties.

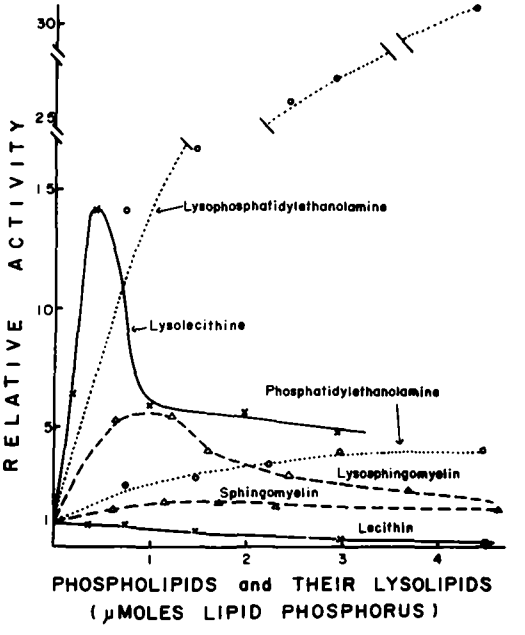

FIG. 4. Relative activity of lysophospholipids.

DISCUSSION

The effect of lipids on heme synthesis was demonstrated. Recently Neuberger et al. reported the effects of lipids and solvents on

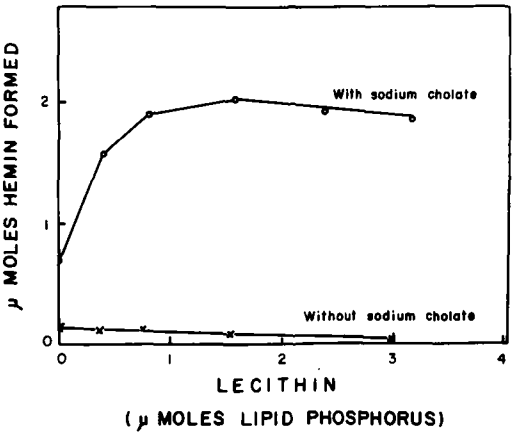

FIG. 6. Effect of lecithin on enzyme.

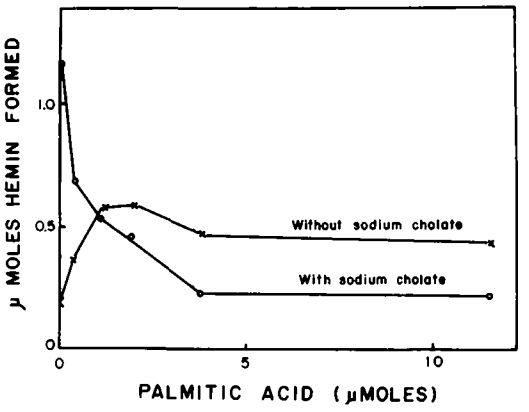

FIG. 7. Effect of palmitate on enzyme.

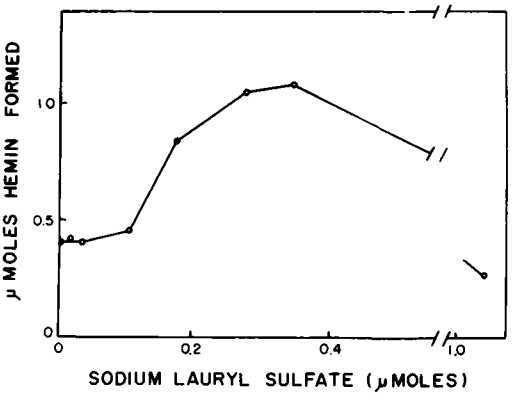

FIG. 8. Effect of sodium laurylsulfate on enzyme.

the formation of zinc protoporphyrin and heme by *Rhodopseudomonas spheroides* chromatophores and by guinea pig mitochondrial preparations (11). They tested several purified lipids and reported that phosphatidic acid was effective and lecithin was ineffective. Using a wider selection of purified lipids, the authors reached the conclusion that the structure of lipids have some definite relationship with the protoheme ferrolyase activity.

Our results with acidic phospholipids showed clearly the charge effects of lipids on enzyme activity. High activity of the acidic phospholipids suggests the importance of an anionic phosphoric acid radical. The strong positive charge of choline in lecithin and sphingomyelin diminishes the effect of the phosphoric acid anion. Effects of charges of lipids on phospholipase B activity were extensively studied by Bangham and Dawson (12,13). They showed that the negative charge of the substrate activated the enzyme system. In our system, negative charge of the phospholipid might attract ferrous iron and thus activate the system.

The potent activity of lysolecithin, which contains a positively charged choline residue, might be explained by the favorable detergent effect of the lyso-compound. Its detergent effect may overcome the unfavorable effect of the cationic charge of choline and the hydroxyl group at the β position may cooperate with the anionic charge of phosphoric acid. The same reasoning may also hold for the case of a cationic synthetic detergent, cetylpyridinum bromide.

It is not clear at present whether a single lipid or a combination of lipids were concerned with protoheme ferrolyase. Lysolecithin and lysophosphatidylethanolamine, the most stimulative lipids, were minor lipids in chicken erythrocyte stroma. The activation of enzyme with lyso-compound has been reported for cytochrome oxidase (14), phosphorylcholine cytidyltransferase (15) and glucose-6-phosphatase (16). According to Cohen and Wainio (14), lyso-compounds are more soluble in aqueous media than diacyl compounds and can effectively reach the site of action.

Protoporphyrin is not soluble in neutral aqueous solution, and it would be possible that the hydrophobic groups of phospholipid molecules, as a part of lipoprotein protoheme ferrolyase, attract porphyrin and reject water molecules. According to Wang and Fleischer (17), the rate of non-enzymic combination of metal ions with porphyrins was strongly solvent-dependent. For the porphyrin molecules to "swallow" a metal ion, porphyrin must peel off most of the solvated ion. In the enzymatic reaction, the hydrophobic part of the lipid molecule might play the role of a favorable solvent. The hydrophilic phosphoric acid group would attract ferrous ion, which is further transferred to the hydrophobic region of lipids where water molecules are stripped off the solvated metal ion and it is "swallowed" by the protoporphyrin molecule.

The high activity of lecithin, and to a lesser degree of sphingomyelin in the presence of cholate, may explain the high activity of the cholate extracted enzyme. It may also explain the stimulative effects of crude lipids in the presence of cholate on the 0.4 M KCl extracted enzyme, and the decreased effect of egg yolk lipid in the presence of cholate after the treatment with Naja naja venom.

ACKNOWLEDGMENT

Professors Yamakawa, Nojima, Ohno, Hara, Kabara and The Michigan College of Osteopathic Medicine rendered valuable assistance.

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[Received September 24, 1968]

The Quantitative Production of Aldehydes From O-Alk-1-enyl Glycerols

ROBERT E. ANDERSON[1], **RONALD D. GARRETT**[2], **M. L. BLANK** and **FRED SNYDER**,
The Medical Division, Lipid Research Laboratories, Oak Ridge Associated Universities,
Oak Ridge, Tennessee 37830

ABSTRACT

The quantitative recovery and structural alterations of aldehydes liberated from O-alk-1-enyl glycerols on thin layer chromatographic layers have been evaluated by using ^{14}C-labeled fatty aldehydes and chromatography. The results led to the development of a quantitative procedure for isolating the aldehydes produced from O-alk-1-enyl glycerols by shaking an ethereal solution of the ether-linked lipids with concentrated hydrochloric acid. The procedure is rapid and silicic acid does not interfere.

INTRODUCTION

Acid catalyzed hydrolysis of O-alk-1-enyl glycerols to produce alcohols and aldehydes is a well-known reaction.

$$RCH_2\text{-}O\text{-}CH{=}CH\text{-}R' \xrightarrow{H_3O^+} RCH_2OH + R'CH_2CHO \quad [1]$$

Schmid and Mangold (1) utilized this reaction to detect O-alk-1-enyl glycerols on chromatograms. The aldehydes liberated are separated from the reaction products and unreacted starting materials by two dimensional thin layer chromatography (TLC). Schmid and Mangold (2), using neutral plasmalogens from human perinephric fat, demonstrated that aldehydes liberated with HCl fumes were identical in composition to the dimethylacetal derivative of aldehydes prepared with methanolic HCl. Others (3-5) have also used essentially similar procedures to determine the O-alk-1-enyl composition of phospholipids.

We recently applied the separation-reaction-separation technique (1) to a radioactive mixture of lipids (containing O-alk-1-enyl glycerols) that had been reduced with lithium aluminum hydride (6). Contrary to expectation, we observed that most of the radioactivity associated with the alk-1-enyl ethers remained at the origin after chromatography on thin layers instead of migrating with the aldehyde standard. These data led us to investigate the quantitative release of aldehydes from O-alk-1-enyl glycerols by acid hydrolysis in the absence and presence of silicic acid. This paper describes the results of our study.

MATERIALS AND METHODS

Octadecenal was prepared by oxidation of the tosylate derivative of octadecenol in dimethylsulfoxide and sodium bicarbonate according to the procedure of Mahadevan et al. (7). The 1-^{14}C-hexadecanal was made from 1-^{14}C hexadecanol by the same procedure (7). Both aldehyde preparations were purified by preparative chromatography on 0.5 mm Silica Gel G thin layers in an equilibrated solvent system of hexane-ether (80:20 v/v).

Total lipids of rat brain were reduced with lithium aluminum hydride by a microprocedure (6) that evolved from earlier experience with $LiAlH_4$ (8,9). The resulting O-alk-1-enyl glycerols were obtained by preparative chromatography on 0.5 mm thin layers of Silica Gel G in an equilibrated solvent system of ether-water (100:0.5 v/v). Isopropylidene derivatives of the O-alk-1-enyl glycerols were prepared according to the procedure of Wood (10), a scaled-down modification of that described by Hanahan et al. (11). The isopropylidenes were resolved by gas liquid chromatography (GLC) conditions described elsewhere (12). The intact aldehydes were analyzed quantitatively by GLC according to the procedure of Wood and Harlow (13). Solvents and other chemicals were reagent grade and used without further purification.

RESULTS AND DISCUSSION

We evaluated the quantitative production of aldehydes liberated by the procedure of Schmid and Mangold (1) by pipetting an aliquot of 1-^{14}C-hexadecanal containing 50 μg octadecenal at the bottom of thin layers of Silica Gel G and exposing the adsorbent to HCl fumes according to their specifications (1). After development in three separate solvent systems, the plates were subjected to zonal profile scan analysis (14). The results are shown in Figure 1; the scans represented by broken lines indicate the purity of the starting radioactive aldehyde (control) and those scans represented with solid

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[1]Present address: Department of Ophthalmology, Baylor University College of Medicine, Houston, Texas 77025.

[2]Present address: College of Pharmacy, University of Texas, Austin, Texas 78712.

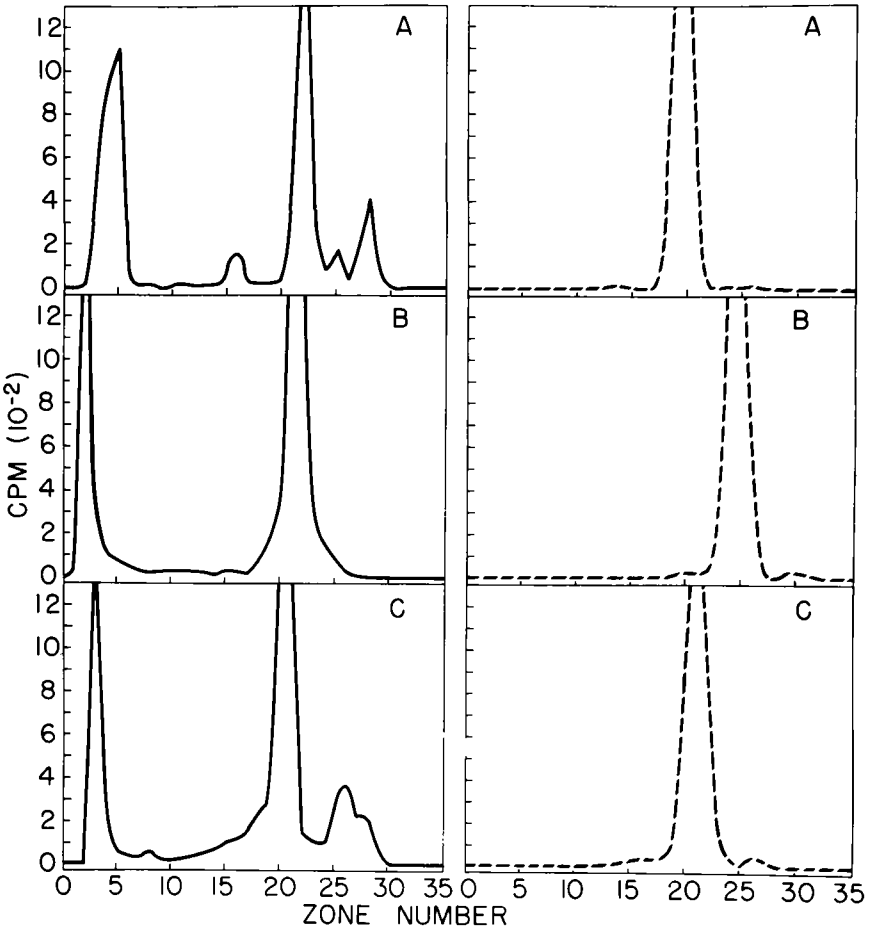

FIG. 1. Zonal profile scans of 1-^{14}C-hexadecanal before (broken lines) and after (solid lines) exposure to HCl fumes according to procedure described in the text. A, developed in hexane-ether (80:20 v/v); B, developed in hexane-ether-acetic acid (60:40:1 v/v/v); and C, developed in hexane-ether-ammonium hydroxide (60:40:1 v/v/v).

lines depict the ^{14}C-activity associated with aldehydes after exposure to HCl fumes (1). Exposure to fumes caused approximately one half of the radioactivity to remain at the origin in each of the three solvent systems; similar data were obtained in repeated experiments in which we consistently found 50-85% of the radioactivity to be localized in regions other than that of the aldehyde. We cannot explain the erratic response since all reaction products were not identified.

The procedure of Viswanathan et al. (5) is similar to that of Schmid and Mangold (1), except that the plate exposed to HCl fumes is neutralized by subsequent exposure to NH_3 vapor before chromatography. We treated 1-^{14}C hexadecanal and 50 μg octadecenal by the method of Viswanathan and coworkers (5) and found only 42% of the activity on the TLC plate to be in the aldehyde region. No attempt was made to chromatograph the aldehydes as their dimethyl acetal derivatives (4).

The following procedure was developed in our laboratory for the production of aldehydes from lipid mixtures containing O-alk-1-enyl glycerols. A solution of O-alk-1-enyl glycerols (0.1-2 mg) in 1.5 ml ether is placed in a 1 dram vial and 1 ml of concentrated HCl is added. After vigorous shaking for 1 to 2 min, the ether layer is removed and the aqueous phase extracted once with ether and once with hexane. The combined ether extracts are washed twice with distilled water to remove all traces of acid before they are evaporated to dryness under a stream of nitrogen. The reaction products are redissolved in a small amount

TABLE I

Composition of Aldehydes Obtained From O-Alk-1-enyl Glycerols After Various Treatments[a]

Sample	Aldehydes[b] 16:0	17:0	18:0	18:1	Total 18
			Area per cent		
1) O-alk-1-enyl glycerols (Isopropylidene derivative after hydrogenation)	23.3	1.1	75.6		75.6
2) Aldehydes liberated with HCl fumes	22.2	1.1	42.9	33.8	76.7
3) Aldehydes liberated by shaking with HCl in the absence of silica gel	22.7	1.1	42.9	33.2	76.1
4) Aldehydes liberated by shaking with HCl in the presence of silica gel	22.2	1.1	42.7	34.0	76.7

[a]O-Alk-1-enyl glycerols, prepared from rat brain, were hydrogenated and then converted to isopropylidene derivatives for GLC; 2) sample exposed to HCl fumes on a TLC plate; 3) and 4) samples shaken in ether with concentrated HCL.

[b]Only traces (< 0.1%) of material with retention times longer than 18:1 were found.

of ether and the aldehydes are recovered by preparative TLC in an equilibrated system of hexane-ether (80:20 v/v). The reaction products recovered after a typical hydrolysis are shown in Figure 2. The minor component (< 5% of total mass) on the chromatoplate appearing midway between the O-alk-1-enyl glycerols and liberated aldehydes is probably the cyclic acetal of the O-alk-1-enyl glycerols.

This procedure was also shown to be quantitative in terms of aldehyde recovery after shaking an ethereal solution of 1-^{14}C-hexadecanal and 150 μg O-alk-1-enyl glycerols with concentrated HCl for 1 min. Zonal profile scans indicated that the aldehydes were structurally unaltered by this procedure. Furthermore, results obtained in which the hydrolysis was carried out in the presence of 50 mg Silica Gel G indicate that O-alk-1-enyl glycerols separated by preparative TLC can be hydrolyzed directly to aldehydes in the presence of Silica Gel G, thereby eliminating the time-consuming elution and concentration step.

The composition of the aldehydes liberated from O-alk-1-enyl glycerols by the separation-reaction-separation procedure (1) and by shaking of an ethereal solution of O-alk-1-enyl glycerols with concentrated HCl in the presence and absence of silicic acid was determined by gas liquid chromatography (Table I). These data were compared to the composition of the isopropylidene derivatives of the O-alk-1-enyl glycerols that had been hydrogenated with platinum oxide catalyst. The C-16 and C-18 aldehydes produced by either type of hydrolysis have identical compositions, and they are identical to the ether-linked hydrocarbons of the hydrogenated starting materials, in agreement with earlier data of Schmid and Mangold (2). We conclude that aldehydes produced by hydrolyses of O-alk-1-enyl glycerols with acid

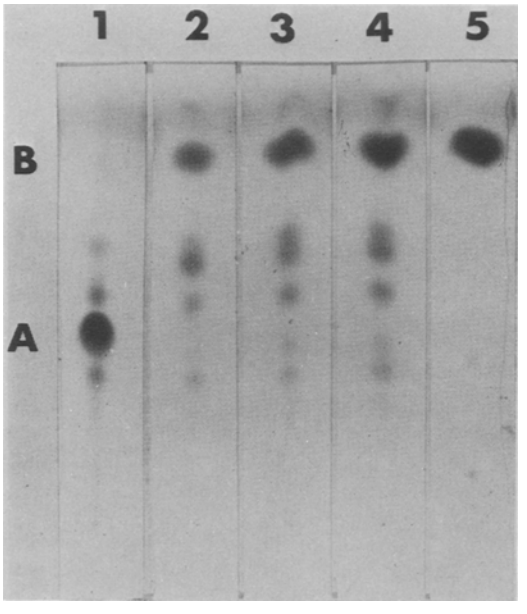

FIG. 2. Thin layer chromatography of O-alk-1-enyl glycerols and their hydrolytic products after shaking in ether with concentrated HCl for various periods of time. A, O-alk-1-enyl glycerols; B, aldehydes; lane 1, O-alk-1-enyl glycerols; lanes 2, 3 and 4, reaction products after shaking 1, 2 and 3 min with concentrated HCl, respectively; and lane 5, standard octadecenal. Developing solvent, ether-water (100:0.5, v/v).

fumes directly on chromatoplates or by shaking ethereal solutions of silicic acid scrapings with concentrated HCl are identical in composition. However, the latter procedure is essential for quantitative recovery of aldehydes, and it can be directly applied to the analysis of O-alk-1-enyl glycerols in the presence of silicic acid.

ACKNOWLEDGMENTS

Research supported in part by American Cancer Society Grant No. P-470 and the U.S. Atomic Energy Commission.

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[Received December 26, 1968]

The Role of Lipids in Membrane Transport in *Mycoplasma laidlawii*[1]

PAUL F. SMITH, Department of Microbiology,
School of Medicine, University of South Dakota, Vermillion, South Dakota 57069

ABSTRACT

Pulse labeling of metabolizing cells of *Mycoplasma laidlawii*, strain B with ^{14}C-glucose results in rapid turnover of the glucose moiety in carotenyl glucoside and phosphatidyl glucose. Pulse labeling also occurs in the fatty acid radicals of carotenyl esters. No labeling of phosphatidyl glycerol, carotenol or monoglucosyl diglyceride occurs. No turnover of ^{14}C occurs in diglucosyl diglyceride. Rapid turnover of ^{32}P in phosphatidyl glucose, but not in phosphatidyl glycerol, also occurs. The rate of $^{14}CO_2$ evolution is equivalent to the rate of disappearance of ^{14}C in total lipids of cells prelabeled with ^{14}C-glucose. β-Glucosidase inhibition results in inhibition of glucose utilization. The reverse effect is not seen. A theory involving lipids as carriers in the transport of glucose into the cell and acetate, the end product of glucose metabolism, out of the cell is presented. This theory is compatible with known information concerning membrane structure, membrane transport and metabolism in Mycoplasmas.

INTRODUCTION

Microorganisms belonging to the genus Mycoplasma afford an excellent model system for the study of membrane composition and function. Despite their small size (250 mμ), they are capable of growth on relatively defined media, multiply rapidly and contain but one membranous structure, the envelope surrounding the organism. This membrane is analogous to all cytoplasmic membranes, being composed almost entirely of lipoprotein and possessing a unit membrane structure. The mycoplasmas are completely devoid of the usual rigid outer wall found in other microorganisms and plant cells (1). The easily isolable membranes can be disaggregated by anionic detergents to yield ultracentrifugally homogeneous subunits (2,3). These subunits contain all of the lipid of the organism together with well defined enzymic activities. One species, *M. laidlawii*, strain B, has been singled out for detailed study because of the ease of its cultivation and of the preparation of membranes.

The lipids of *M. laidlawii* constitute 8-12% of the dry weight of the organisms and are equally divided into neutral and phospholipids (1). The neutral lipids are composed of an all *trans* C_{40} 3,3'-dihydroxy carotene, the exact structure of which has not been elucidated, fatty acid esters of this carotenol in which acetic acid predominates, and a β-D-glucoside of the carotenol (4). Small amounts of neurosporene and trace amounts of phytoene, phytofluene and ζ-carotene also are present. The remainder of the neutral lipids are made up of O-α-D-glucopyranosyl-(1→1) diglyceride and O-α-D-glucopyranosyl-(1→2)-O-α-D-glucopyranosyl-(1→1) diglyceride (5). These glucosyl diglycerides constitute up to 45% of the total lipid. The phospholipids found in this organism are phosphatidyl glycerol and diacyl α-glycerophosphoryl-1-(α,β)-D-glucose (5,6).

There exist among the mycoplasmas species which utilize glucose for a carbon and energy source, and species which, being incapable of hexose degradation, utilize fatty acids (7). *M. laidlawii* belongs to the former group (1). Only in this group are glycolipids found. These organisms ferment glucose by the classic Embden-Mayerhof scheme resulting in the accumulation of acetate as the end product (8-10). The occurrence of glucose and acetate as primary constituents of certain lipids suggested that these lipids may be involved in the utilization of glucose. The membranous location of the lipids further intimated that their role may be the transport of glucose into the cell and of acetate out of the cell (11). Many membrane transport phenomena appear to require carriers to mediate traversal of the lipid barrier. Although a few proteins have been isolated from osmotic shock fluids of bacteria and are thought to represent carriers because of their binding properties for specific ions (12) or compounds, membrane transport carriers are extremely elusive.

The hypothesis that certain lipids of *M. laidlawii* behave as carriers for glucose and the end product of its metabolism, acetate, led to a search for enzymes capable of attaching and

[1]Presented at the AOCS Meeting, Chicago, October, 1967.

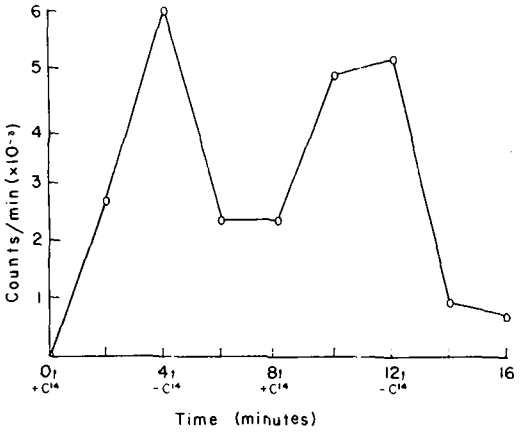

FIG. 1. Appearance of exogenous ^{14}C labeled acetic acid during metabolism of glucose. Exogenous ^{14}C-glucose added or removed as indicated by arrows.

removing these radicals. An esterase capable of hydrolytic or thiolytic cleavage of steryl or carotenyl esters is found in the cytoplasmic membrane. Synthesis of acetate esters of hydroxylated polyterpenes also occurs in the membrane (13). These activities are distinguishable from lipase activity which is found in the soluble cell fraction or extracellularly. A membrane-associated glucosidase occurs in all of the fermentative mycoplasma examined but never in the non-fermentative species (14). This glucosidase exhibits narrow specificity for glucose and preferentially attacks glucosides with aryl aglycons. In the case of *M. laidlawii*, its anomeric specificity is β. Studies are in progress to define the mechanisms for the synthesis and degradation of the carotenyl glucoside, phosphatidyl glucose and the glucosyl diglycerides.

A critical factor in establishing a carrier role for lipids in the transport of glucose and acetate is the rate of turnover of these radicals in the lipids. This report is a compilation of our current knowledge in this area.

EXPERIMENTAL PROCEDURES

Turnover of ^{14}C-glucose in Neutral Lipids

Logarithmic phase organisms were subjected to pulse labeling with uniformly labeled ^{14}C-glucose (15). Samples were removed at 2 min intervals. The supernatant fluid was extracted at pH 4 with diethyl ether to determine exogenous acetate. The sedimented cell pellet was extracted with chloroform-methanol and the various species of lipids quantitatively separated by column and thin layer chromatography (6). Figure 1 demonstrates that the appearance of exogenously labeled acetate follows the same course as the availability of exogenously labeled glucose. These data provide evidence that the glucose is being metabolized to acetate. The labeling patterns of the carotenyl glucoside and the carotenyl ester are shown in Figure 2. The carotenyl glucoside becomes labeled slightly more rapidly than the carotenyl ester. Both lipids rapidly lose radioactivity upon removal of exogenous ^{14}C-glucose. This phenomenon occured over two complete pulses. Although it would be anticipated that the glucoside should become labeled prior to the ester as it appears in the first pulse, no significance is attached to this result because the techniques employed could not differentiate between such rapid changes. As described later deoxycorticosterone and iodoacetic acid inhibit glucose utilization in *M. laidlawii*. As shown in Figure 2, deoxycorticosterone completely abolishes labeling of either lipid, indicating interference with

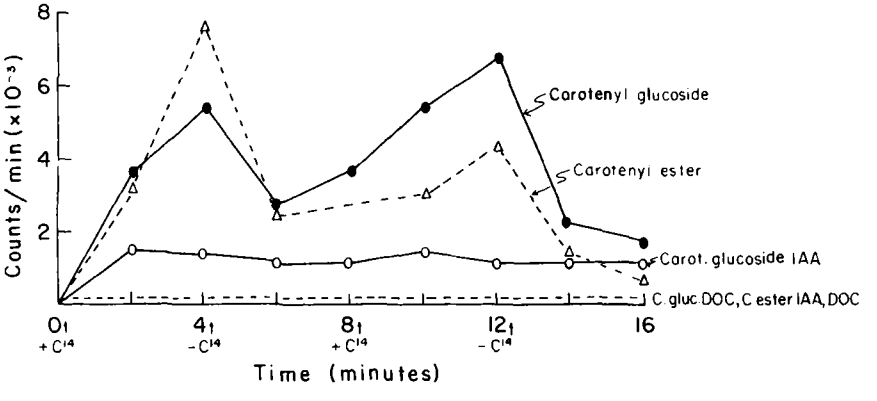

FIG. 2. Radioactivity appearing in carotenyl derivatives during metabolism of glucose with and without inhibition. Exogenous ^{14}C-glucose added or removed as indicated. IAA, iodoacetic acid 5 x 10^{-2} M; DOC, deoxycorticosterone 5 x 10^{-3} M.

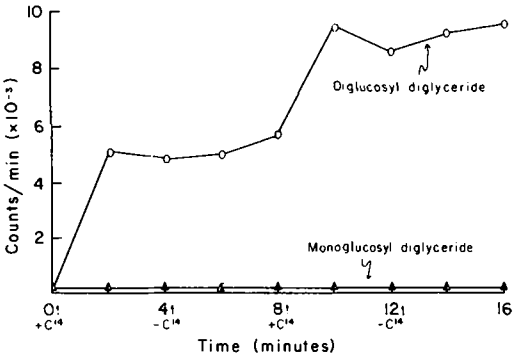

FIG. 3. Radioactivity appearing in glucosyl diglycerides during metabolism of glucose. Exogenous ^{14}C-glucose added or removed as indicated.

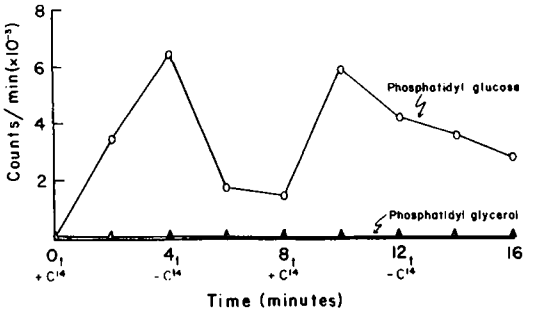

FIG. 4. Radioactivity appearing in phospholipids during metabolism of glucose. Exogenous ^{14}C-glucose added or removed as indicated.

synthesis of the glucoside. Iodoacetic acid allows minimal synthesis of the glucoside but prevents its turnover. This result is compatible with the inhibitory activity of this compound on the β-glucosidase and its known interference with the fermentation of glucose.

No incorporation of ^{14}C-glucose occurs into the monoglucosyl diglyceride. The diglucosyl diglyceride becomes labeled during exposure of the organisms to ^{14}C-glucose but does not lose radioactivity upon removal of the exogenous label. Further incorporation occurs upon readdition of ^{14}C-glucose (Fig. 3). No radioactivity appears in the free carotenol or the hydrocarbon carotenes, proving that the ^{14}C incorporation is not the result of carotenoid synthesis.

Turnover of ^{14}C-glucose and Inorganic ^{32}P in Phospholipids

Metabolism of glucose under conditions of pulse labeling with ^{14}C-glucose gives the same result with phosphatidyl glucose as with the carotenyl glucoside (Fig. 4). It appears that the radioactivity in phosphatidyl glucose is incorporated and removed even faster than in the carotenyl glucoside. No radioactivity is detectable in the phosphatidyl glycerol. Although not shown in the Figure, pulse labeling with inorganic ^{32}P during metabolism of unlabeled or ^{14}C-glucose yields the same labeling pattern as pulse labeling with ^{14}C-glucose alone. Therefore both glucose and phosphate of the phosphatidyl glucose turn over rapidly during glucose metabolism.

Another type of labeling experiment using inorganic ^{32}P was performed to demonstrate phosphate turnover in the phosphatidyl glucose and non-turnover in the phosphatidyl glycerol. Logarithmic phase organisms of *M. laidlawii* from a 400 ml culture were harvested aseptically and incubated in 400 ml 0.2 M pH 7.5 tris (hydroxymethyl aminomethane)-maleate buffer containing 10 mmoles of glucose, 100 μmoles of $MnCl_2$ and 1.5 mc of $KH_2{}^{32}PO_4$ at 37 C. After 15 min the reaction mixture was added to growth medium containing 100 mmoles of pH 7.5 phosphate buffer. Samples were removed at various stages of incubation, organisms harvested and the phospholipids isolated and counted (6). No labeling of the phosphatidyl glycerol occurred during the metabolic phase of the experiment but ^{32}P was incorporated into this lipid during growth of the organisms. The phosphatidyl glucose became highly labeled during the metabolic phase of the experiment and rapidly lost its radioactivity during subsequent growth (Fig. 5). These results offer further evidence that only phosphatidyl glucose of the phospholipids turns over during metabolism and hence probably plays a role in the catabolic utilization of glucose.

Rate of CO_2 Appearance Relative to the Disappearance of ^{14}C in Total Lipids

If glucose and acetate must pass through the cytoplasmic membrane covalently bonded to certain lipids, then the rate of appearance of exogenous $^{14}CO_2$ should approximate the rate of disappearance of ^{14}C in the carotenyl glucoside, carotenyl acetate and phosphatidyl glucose. Since none of the other lipids, with the exception of diglucosyl diglyceride, become labeled during metabolism of ^{14}C-glucose, disappearance of radioactivity in the total lipid fraction only was measured together with the evolved CO_2 which was trapped in $Ba(OH)_2$. Organisms (0.3 g dry wt) were allowed to metabolize ^{14}C-glucose (500 μmoles; 30 μc) for a period of 10 min after which the exogenous glucose was removed by repeated washing of the organisms by centrifugation. The organisms then were allowed to metabolize unlabeled glu-

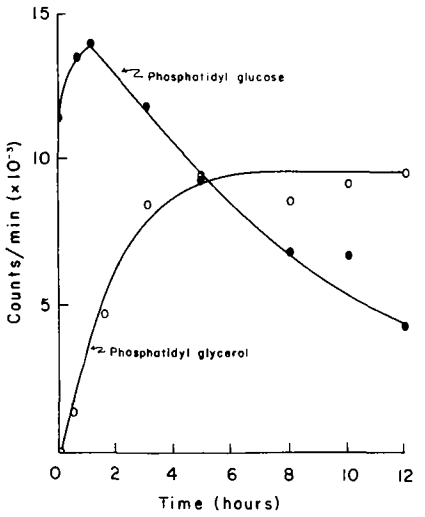

FIG. 5. Turnover of ^{32}P in phospholipids during growth following exposure to ^{32}P during metabolism of glucose.

cose, samples of the trapped CO_2 and the organisms being taken at 2 min intervals. Total lipids were isolated by chloroform-methanol extraction followed by purification by passage through sephadex columns. CO_2 was counted as $BaCO_3$. Figure 6 shows that the rates of $^{14}CO_2$ evolution and the disappearance of ^{14}C from the total lipids are approximately the same. Glucose and acetate turnover in the lipids in question is as rapid as catabolism of glucose.

Inhibition of Glucose Utilization

The behavior of certain lipids as possible membrane transport carriers for glucose and acetate would be dependent upon the proper functioning of enzymes associated with the synthesis and degradation of carotenyl glucoside, carotenyl ester and phosphatidyl glucose. Although inhibitory compounds usually lack well-defined specificities, the action of certain

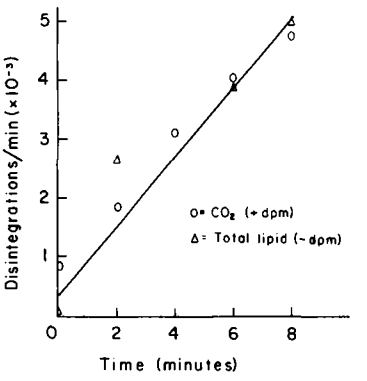

FIG. 6. Rates of appearance of $^{14}CO_2$ and disappearance of ^{14}C from total lipid during metabolism of glucose following prelabeling with ^{14}C-glucose.

inhibitors can provide corroborative evidence. Thus far, our efforts in this type of analysis have been directed mainly toward the β-glucosidase and overall glucose utilization (14). A series of compounds have been examined for their ability to inhibit the β-glucosidase and to prevent the utilization of glucose (Table I). All of the selected inhibitors prevent glucose utilization while only two, iodoacetic acid and uranyl nitrate, are really effective against the glucosidase. It should be noted that in all instances, though limited in number, compounds which inhibit glucosidase activity also prevent glucose utilization. These results are compatible with the effects on glucose turnover as shown in Figure 2.

DISCUSSION

Definitive proof that certain membrane components act as transport carriers is difficult if not impossible to obtain. At best most evidence is circumstantial, as is the foregoing data. Nevertheless, it is tempting to postulate a membrane permeation system for glucose and its end product of metabolism, acetate, involving

TABLE I

Effect of Inhibitors on Glucose Utilization and β-Glucosidase Activity of *Mycoplasma Laidlawii*

Compound	Concentration	Effect on Glucose utilization[a]	Effect on β-Glucosidase[a]
Deoxycorticosterone	5×10^{-3} M	+	0
Estradiol	5×10^{-3} M	+	0
N-Ethylmaleimide	5×10^{-3} M	+	±
Iodoacetic acid	5×10^{-2} M	+	+
Uranyl nitrate	5×10^{-2} M	+	+
Phloridzin	5×10^{-3} M	+	0

[a] +, inhibition; ±, partial inhibition; 0, no inhibition.

lipid carriers. A diagrammatic representation of a structure for the cytoplasmic membrane of mycoplasmas taking into account all of our present information on the subject has been proposed. Likewise, permeability mechanisms which could function in such a model have been proposed and summarized (16). The permeability mechanism set forth here fits the aforementioned model but applies only to glucose and acetate transport.

The membrane is envisioned as a series of distinct lipoprotein globules held together by phospholipid bridges. The protein of each globule is coiled so that the apolar segments are located in the interior and the polar segments at the exterior. Each globule may contain one or more enzyme or carrier proteins. The intact membrane would be a mosaic of proteins differing in enzymic composition but similar in mass and dimension. There is evidence that specific enzymic activity is scattered, not diffuse, in mycoplasmal membranes. The fatty acid residues of glycerophospholipids are bound by hydrophobic bonds into the apolar segment of coiled protein. The glycerophosphate segments lie external to the protein and form the bridge between subunits, best illustrated with (bis) phosphatidyl glycerol. Lying between the negatively charged oxygen atoms on the phosphate radicals are divalent cations, e.g., Mg^{++}. The presence of the cation tends to pull the subunits close together because of the condensation of the lipid molecule. In the condensed membrane, pores of sufficient size exist to allow free entry and exit of such molecules as water. However, passage would be governed by the nature of the areas lining the pore. To permit specific entry of other molecules, the cation could be removed as a result of its requirement for enzymic activity, resulting in expansion of a specific membrane area or areas and the subsequent formation of larger pores. The subunits remain attached and merely spread apart at given locations. The specific enzyme or carrier protein at these sites would regulate what passes into or out of the cell.

In the case of glucose transport into the cell the enzymes associated with the synthesis and degradation of carotenyl glucoside or phosphatidyl glucose would be found in the subunits surrounding the pore. In the case of acetate exit from the cell, the enzymes of the subunits would be involved in the synthesis and degradation of carotenyl acetate. In order to include the carotenol and the phosphatidyl glucose in the permeability scheme, it is necessary to place them in some oriented manner in the membrane subunits. This is easily accomplished by having their apolar segments bound in the hydrophobic area of the protein coil. For example, the dihydroxy carotenol would lie lengthwise accross the width of the subunit, one hydroxyl group directed to the exterior, the other to the interior of the cell. Such an orientation is reasonable since the lengthwise dimension of the apolar segment of the carotenol and the width of the inner electron non-absorbant area of the mycoplasmal membrane approximate 30 A. In this orientation, the hydroxyl group facing the exterior of the cell would be available for the covalent attachment of glucose, the hydroxyl group facing the interior of the cell for the attachment of acetate. The change in polarity resulting from the attachment of glucose (increase in polarity of the lipid) and acetate (decrease in polarity) could trigger a rotational movement of the subunit to the interior of the cell (glucose) or to the exterior of the cell (acetate) where the appropriate enzyme could release the glucose or acetate. The carotenol freed of its radicals would be in the proper orientation to repeat the process. The phosphatidyl glucose, of course, could serve only in the transport of glucose into the cell. The movements in the membrane resulting from the opening and closing of pores and the rotational movements of subunits could explain the fibrilliations observed in membranes of metabolizing cells.

This postulate on how molecules are specifically transported into and out of cells sounds too simple. It does fit all the data presently available for the mycoplasmas, even those which contain no carotenoids but require sterols for growth, a subject not discussed in this report. However, attempts to apply the role invoked for lipids to membrane transport in other cells brings about some incompatibility (17). Nevertheless, the basic principles may be the same. The lipids to which are ascribed specific carrier functions in mycoplasmas have not been described in all other cells. Other lipids conceivably could carry out similar functions. Membrane transport of glucose in other microorganisms is thought not to involve covalent bonding to a carrier. In these instances carrier protein lining a pore could achieve the same function. Mycoplasmas may differ from other cells in certain transport phenomena because bacterial and plant cells are surrounded by rigid walls as well as cytoplasmic membranes and the membranous structures of animal cells are not quite as simple as a single enveloping unit membrane. Intracellular glucose in other microorganisms appears as free glucose not as a phosphorylated glucose. Such could be the case with glucose transported through the mediation of the carotenol but one would anticipate that

phosphatidyl glucose mediated transport would result in intracellular glucose-1-phosphate. Compatibilities exist where lipid is not involved as a carrier and with respect to specificity and kinetics of transport.

Doubtless the proposed scheme is oversimplified and is subject to change or disproval. It does offer a model upon which further insight may be gained into the structure and function of cytoplasmic membranes, their lipids, and their enzymes.

ACKNOWLEDGMENTS

This study was supported in part by research grant AI-04410-06 from the National Institute of Allergy and Infectious Diseases.

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[Received September 27, 1968]

Flotation Rates, Molecular Weights and Hydrated Densities of the Low-Density Lipoproteins[1]

F. T. LINDGREN, L. C. JENSEN, R. D. WILLS and N. K. FREEMAN, Donner Laboratory, Lawrence Radiation Laboratory, University of California, Berkeley, California 94720

ABSTRACT

A method involving three computer programs is described for characterizing the major component of the S_f 0-12 low-density lipoprotein class by its S_f° rate, hydrated density and molecular weight. All necessary information is obtained from a standard low and high-density lipoprotein ultracentrifugal analysis. Moving-boundary flotation rates are measured in 1.061 g/ml sodium chloride and 1.200 g/ml sodium bromide solutions and are corrected to flotation at zero concentration. Hydrated densities are calculated from η F° versus ρ plots and minimum hydrated molecular weights calculated using Stokes' frictional factor, assuming spherical molecules. Preliminary application of this procedure indicates higher S_f° rates, higher molecular weights, and lower hydrated densities in females than in males. Molecular weights and standard deviations of the principal S_f 0-12 component for non-fasting normal adult females and males were 2.36 ± 0.16 and 2.12 ± 0.20 millions, respectively.

INTRODUCTION

Several studies have indicated that the principal low-density S_f 0-12 lipoprotein class represents a distribution in size, hydrated density and chemical composition (1-3). The content of protein (1) appears to be the major factor determining hydrated density of this class, which ranges from approximately 1.02-1.05 g/ml. Values given for molecular weights of this class have ranged from 1.3-3 million (4-8) and depend, in part, on the techniques employed and the portion of the lipoprotein distribution studied. Because of the very nature of this distribution of values, for specific characterization it is necessary to focus on the most abundant S_f 0-12 component as measured in the analytic ultracentrifuge. The flotation rate of this major low-density component varies from individual to individual and has a range of about S_f° 4-8 Svedberg units. Normal females have S_f° rates approximately 1 S_f° unit faster than normal males (9,10). There also appear to be unusual flotation rates of this component associated with specific lipid and lipoprotein abnormalities (11).

Although computer techniques have been described for analysis of lipoprotein distributions (12) and for precision moving-boundary flotation rate determinations (9), neither molecular weight nor hydrated density determinations based on standard low and high-density lipoprotein analysis have been presented. Theoretically, there is sufficient information available from these runs, made simultaneously, to make such calculations. Our present method utilizes these two computer programs. All S_f°, σ and molecular weight calculations are made using a third, separate computer program.

METHODS

The normal male and female subjects of this study were clinically healthy employees of Lawrence Radiation Laboratory at Livermore and Berkeley, California. Serum was prepared from mid-morning nonfasting blood specimens. A complementary series of fasting male and female clinical referrals were obtained from Kaiser Hospital, Oakland, California. Each population set was matched for age and grossly overweight or underweight subjects were excluded. Unless otherwise indicated all studies were done using serum containing 1 part/10,000 Merthiolate.

In the normal non-fasting series studied, total low-density lipoprotein (LDL) fractions, $1.006 < \sigma < 1.063$ g/ml, (S_f 0-20) were isolated by preparative ultracentrifugation and total lipids were extracted as previously described (12). Unless otherwise indicated, all densities are given at 26 C. Phospholipid, cholesteryl ester and glyceride composition were determined by infrared spectrometry (13).

Ultracentrifugal lipoprotein fractionation and analytical ultracentrifugal analysis were patterned after earlier techniques (14,15). The computer program and details of preparative ultracentrifugation are described elsewhere (12). All analytic runs were made at 25.5-26 C in modified (16) Beckman Model E ultracentrifuges. A total of 10 schlieren photographs were taken, 1 during acceleration and 9 during the 64 min. up-to-speed (UTS) run at 52,640 rpm. Figure 1 shows selected schlieren photographs

[1]Presented at the AOCS Meeting, New York, October, 1968.

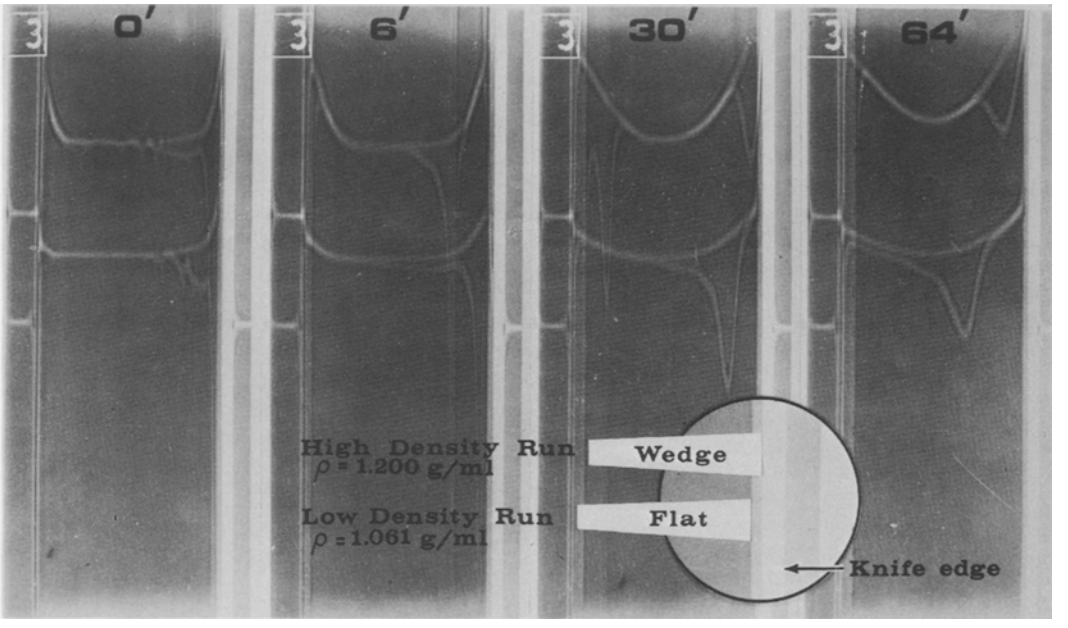

FIG. 1. Selected schlieren photographs from a typical standard low and high-density lipoprotein analysis (Case 1710).

of a total low and high-density lipoprotein run. The 0, 6 and 30 min photographs were used for the low-density analysis and the 64 min photograph used for high-density analysis. In order to unambigously determine the base-of-cell for each run, a 0.020 in. offset centerpiece was used in the flat cells. Classical moving-boundary flotation rates of the major low-density S_f° 0-12 component measured in both 1.061 g/ml sodium chloride and 1.200 g/ml sodium bromide were calculated from a ln x versus $\omega^2 t$ plot using a second computer program (9). Normally, in 1.200 g/ml sodium bromide this component was measured in the 0, 2, 6, 8, 14, and 22 min (UTS) frames; and in 1.061 g/ml sodium chloride it was measured in the 8, 14, 22, 30, 48 and 64 min (UTS) frames. Boundary positions in all cases were determined by the maximum ordinate technique (17). A third computer program, using data and results from the first two programs, performed all the remaining calculations. Flotation rates were corrected for concentration dependence (12) by the standard relationship $F = F^\circ(1\text{-}KC)$ where $K = 0.89 \times 10^{-4}$ (mg/100 ml)$^{-1}$ and C is the concentration in the cell integrated up to the low-density S_f° 0-12 peak position, averaged over the time interval used in the moving-boundary flotation-rate measurement. This average is approximated by a Moring-Claesson type (18) correction: $C = C_o (X_{BC})^2/(X_1 X_2)$ where C_o is the initial base-of-cell concentration as determined in the first analytic ultracentrifuge computer program, X_{BC} is the base-of-cell radial distance, and X_1 and X_2 are the first and last peak positions measured. In the high-density run, C is equal to the sum of both the above low-density concentration and the total high-density concentration, similarly corrected. Background densities of the lipoprotein infranatants were measured indirectly by precision refractometry at 26.0 C. These values and their calculated viscosities each were extrapolated to the corresponding supernatant fraction (corrected for redistribution of salt during the preparative run) and corrected to the mean temperature of the analytic run. From these data a ηF° versus ρ plot was made and a ρ intercept calculated. S_f° rates corrected to standard conditions were made according to the relationship: $S_f^\circ = (\rho_S\text{-}\sigma)(\eta)/(\rho\text{-}\sigma)\eta_S$ where standard values at 26 C for 1.744 molal NaCl were ρ_S = 1.0630 g/ml and η_S = 1.0260 cp.

Assuming Stokes' frictional factor for spheres, a molecular diameter was calculated from the familiar relationship: $S_f^\circ = d^2(\rho_s\text{-}\sigma)/18\eta = d^2(1.0630\text{-}\sigma)/184.7$ where d had been converted to Angstrom units in the final expression. Finally, a minimum hydrated molecular weight was calculated, assuming spheres, from the molecular volume, Avogadro's number and the hydrated density (the latter closely approximated by the density of zero migration or ρ intercept): mol wt (daltons)

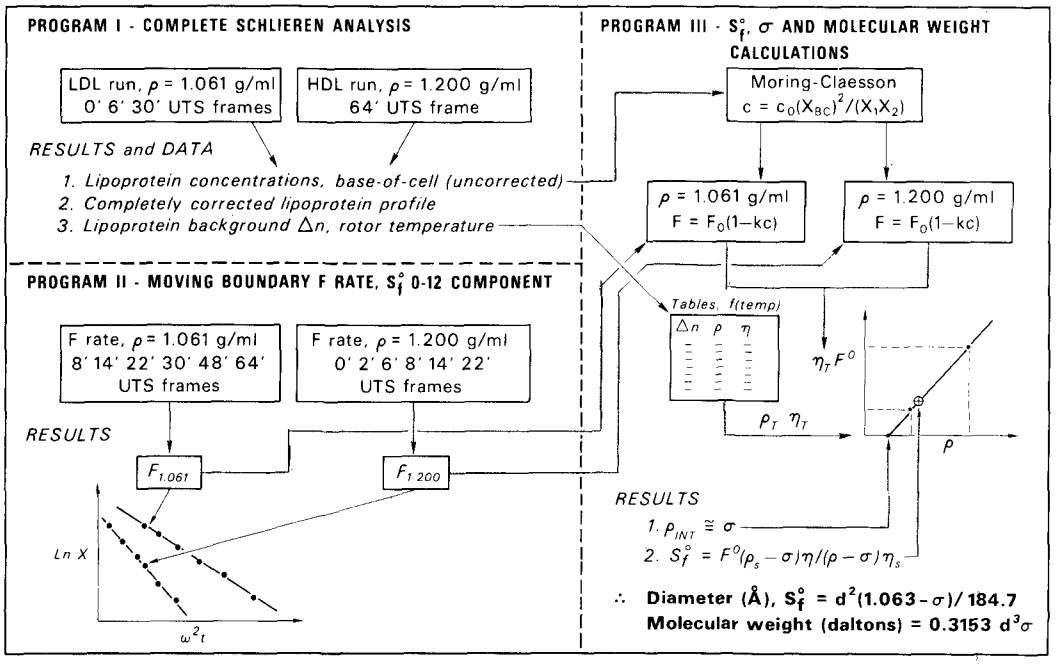

FIG. 2. Schematic diagram showing utilization of the results and data from the three computer programs.

$= \pi d^3/6(10^{-8}cm/A)^3\ N_o\ \sigma = 0.3153\ d^3\sigma$. The relationships between the three computer programs and their data are shown in Figure 2.

Narrow-band serum lipoproteins from a single non-fasting adult male were prepared by an approach-to-equilibrium sodium bromide density gradient technique (2). Fractions were isolated on a density gradient (18 C, 50.3 rotor and 50,000 rpm for 48 hr) corresponding to approximately 1.031, 1.034 and 1.037 g/ml. These isolated lipoprotein fractions were manipulated to 1.061 sodium bromide and 1.200 sodium bromide by appropriate solid sodium bromide addition and densities were monitored by precision refractometry. For density calculations, the refractive increment of these lipoproteins was assumed to be 0.00154 and 0.00140 Δn/g/100 ml in the two media, respectively. Again, flotation rates were calculated and corrected to zero concentration. For this calculation the time dependent total component concentration was used in the manner described earlier. Again, an ηF° versus ρ plot was made yielding ρ intercept values and corrected S_f° rates were calculated. In these studies the problem of correcting for concentration dependence of the low-density component in the presence of a time dependent high-density lipoprotein concentration (different for each individual) was avoided. Here ρ intercept values are independent of the concentration dependence K factor, although S_f° and molecular weight values are affected. For this series, both Merthiolate and non-Merthiolate serum samples were studied, since we routinely use this preservative. However, no significant differences were observed between these two series.

RESULTS AND DISCUSSION

To evaluate technical reproducibility, nine serum aliquots of a single nonfasting normal male subject were completely analyzed by our procedure employing the total low and high-density lipoprotein fractions. A measure of errors are the standard deviation of the results which are as follows: S_f rate = 4.87 ± 0.08 Svedbergs (10^{-13} cm/sec/dyne/g); σ hydrated ≅ ρ intercept = 1.0360 ± 0.0003 g/ml (26 C); molecular weight = 1.984 ± 0.021 (millions). Using non-fasting serum obtained earlier from this same male subject, orthodox ηF° versus ρ studies were made on isolated narrow-band LDL components and the results compared with our technique. Figure 3 shows schlieren photographs of both the 1.061 g/ml sodium bromide and 1.200 g/ml sodium bromide runs of one of the narrow-band lipoprotein fractions prepared from the 1.034 g/ml density gradient fraction (Merthiolate series).

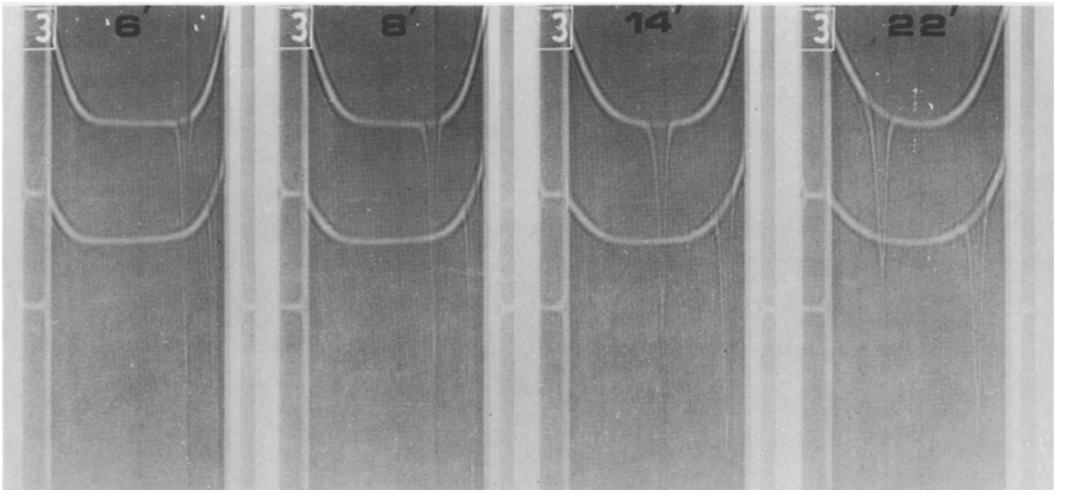

FIG. 3. Schlieren flotation photographs of isolated narrow-band S_f° 6.06 lipoproteins, Case 1710, 52,640 rpm, 26 C; upper wedge cell is ρ = 1.200 g/ml (2.755 molal NaBr, 0.194 molal NaCl), flat cell is ρ = 1.061 g/ml (0.742 molal NaBr, 0.194 molal NaCl). Lipoprotein concentration in the two media is 465 mg/100 ml.

All density gradient samples were analyzed at total component concentrations in the range of 261-704 mg/100 ml. Figure 4 shows the linear regression relationship between S_f° rate and ρ intercept for both the Merthiolate and non-Merthiolate fractions. Also plotted are the results (from the same serum sample) of our procedure utilizing eight standard low and high-density lipoprotein analyses obtained over a period of 33 days. Nine months later nine additional analyses by our method were performed on serum from the same non-fasting subject and the mean value plotted, suggesting minimal biological variation in this person. Figure 5 shows a similar linear regression relationship of S_f° rate and molecular weight. Again, values obtained from simultaneous low and high-density lipoprotein analyses indicate comparable results in the region of the major component compared

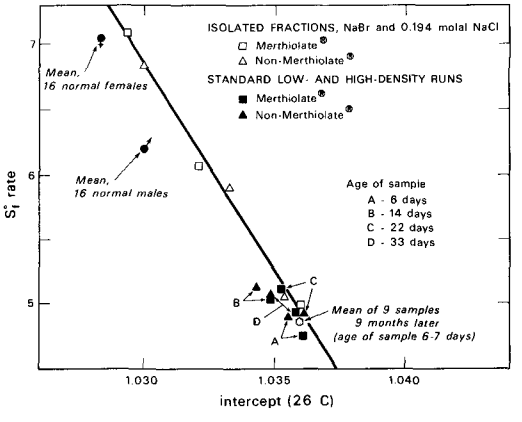

FIG. 4. Relationship between S_f° rate and ρ intercept obtained from isolated narrow-band LDL fractions and from standard low and high-density runs.

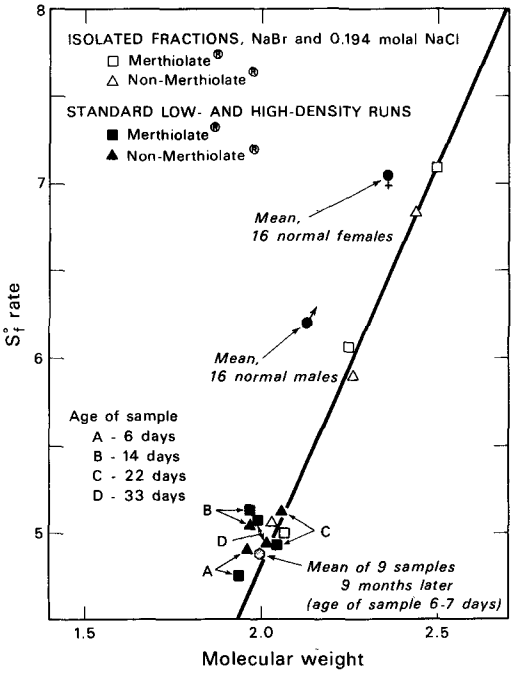

FIG. 5. Relationship between S_f° rate and molecular weight obtained from isolated narrow-band LDL fractions and from standard low and high-density runs.

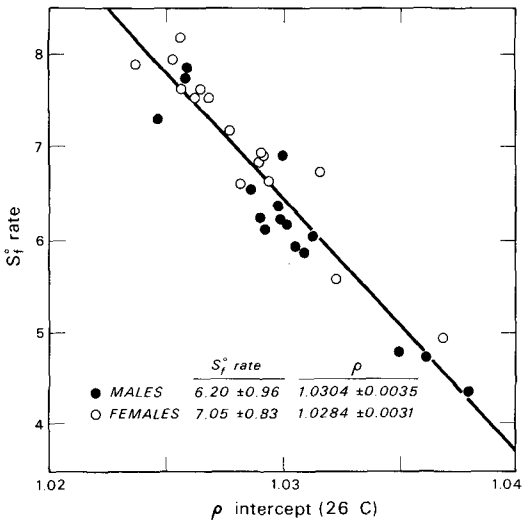

FIG. 6. Relationship between S_f° rate and ρ intercept, normal nonfasting males and females.

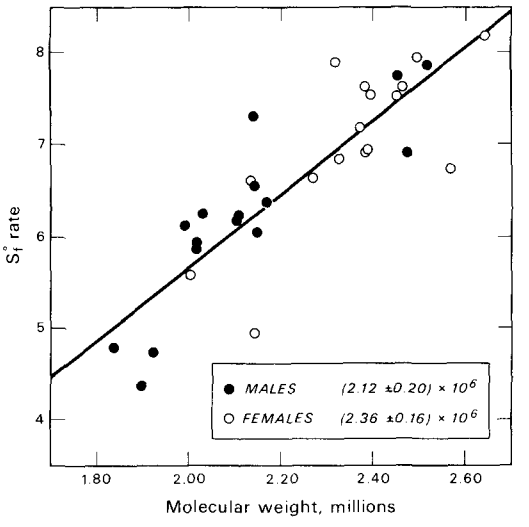

FIG. 7. Relationship between S_f° rate and molecular weight, normal nonfasting males and females.

with the same S_f° rate on the regression line obtained with isolated lipoprotein components. These results suggest our total low and high-density lipoprotein analyses give results closely similar to an ideal procedure. Such a technically difficult method applied to each individual serum would require the identification and narrow-band isolation of the most abundant S_f 0-12 lipoprotein species followed by a classical ηF° versus ρ analysis.

A preliminary application of this method involved nonfasting serum from a small, normal adult, male and female population, ages 35-50 years. Figures 6 and 7 show the relationship observed between S_f° rate and the two calculated parameters, ρ intercept and molecular weight, respectively. Very similar regression is observed with this population as compared with the components isolated from a single normal male individual. For comparison with the isolated component study the mean male and female values are plotted on Figures 4 and 5. At a given S_f° rate there is some variability from person to person in ρ intercept and somewhat more variability in molecular weight. Although the females have significantly higher S_f° rates than the males, there appear to be very similar regression relationships for each population. Table I presents the low-density lipoprotein results for this series of normals, including the correlations between the three variables, S_f° rate, ρ intercept and molecular weight of the major S_f° 0-12 component. The females (compared with the males) have faster S_f° rates, as has been observed before (9,10); they also have approximately a 235,000 higher molecular weight and a slightly lower hydrated density.

Total low-density lipoproteins of the S_f° 0-20 class were isolated from each of the above normal nonfasting males and females. Phospho-

TABLE I

Results, S_f 0-12 Major Component, Normal Subjects, Nonfasting

Age (mean ± SD)	Males (16) 44 ± 3 years	Females (16) 43 ± 4 years	Difference
1. S_f°	6.20 ± 0.96	7.05 ± 0.83	$p < .01$
2. σ (lipoprotein)	1.0304 ± 0.0035	1.0284 ± 0.0031	NS
3. Mol wt (millions)	2.12 ± 0.20	2.36 ± 0.16	$p < .01$
Correlations			
1. S_f° vs σ	-0.95[a]	-0.95[a]	
2. S_f° vs mol wt	0.87[a]	0.76[a]	
3. σ vs mol wt	-0.69[a]	-0.52[b]	

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

TABLE II

Lipid Composition and Lipid σ, S_f 0-20 Lipoproteins, Normal Males and Females[a]

Component[b]	PL	CE	TG	FC	σ(lipid)[c]
Males (n = 16)	24.4 ± 2.3	55.1 ± 1.5	9.4 ± 2.4	11.0 ± 0.3	0.9803 ± 0.0016
Females (n = 16)	26.7 ± 1.8	53.4 ± 1.5	9.3 ± 2.3	10.6 ± 0.3	0.9798 ± 0.0015
Difference	$p < 0.01$	$p < 0.01$	NS	---	NS

[a]Mean and SD values are wt % of total lipid; free cholesterol is assumed to be 0.198 x cholesteryl ester.

[b]PL, CE, TG and FC are abbreviations for phospholipid, cholesteryl ester, triglyceride and free cholesterol.

[c]Calculated assuming additivity of densities for PL, CE, TG and FC; individual values used are 0.97, 0.99, 0.92 and 1.067 g/ml, respectively.

lipid, cholesteryl ester and triglyceride content of the total lipid is presented in Table II. The females have a higher phospholipid and lower cholesteryl ester content in these molecules than the males. However, the differences are small and there is not much variability in lipid composition in these two populations. Although some relationships were observed in the females between these lipid components and the three parameters, S_f° rate, σ and molecular weight, no significant relationships were observed in the male population (see Table III). By calculation, the mean density of the lipid moieties of each population was almost identical. Also, in the females there was a moderately positive correlation of glyceride content with ρ intercept (and a low order positive correlation in the males). These results suggest that the major factor contributing to changes in hydrated density within this class is not glyceride content but is the protein content of the lipoprotein. In a much earlier density gradient study, this relationship between protein content and lipoprotein density was observed on S_f° 3-10 subfractions from pooled human plasma (1).

Another preliminary application of this method was to a small series of 16 male and 19 female clinical referrals from Kaiser Hospital, Oakland, California. These patients were of a wider age range; many had high blood lipids, and in contrast with the normals studied, they were fasting. Table IV presents the low-density lipoprotein results for these populations, including the statistical relationships. Again, as in the normal populations, similar values, differences and correlations were observed. The somewhat lower S_f° rates observed probably reflect a clinical population with higher levels of very low-density lipoproteins (VLDL). Lower S_f° rates would be expected, since in both normal male and female populations there is a significant negative correlation between S_f° rate and VLDL concentration (12).

It is worthwhile to compare our results with a few lipoprotein values obtained earlier by others using different techniques. Bjorklund and Katz (7) compared S_f° rates, hydrated densities and molecular weights of isolated S_f° 4-8 subfractions. However, the molecular weight they obtained by light scattering was considerably higher than our present values, namely, 2.8-3.0 millions. Their data also indicated an axial ratio of 2-2½ to 1. From somewhat similar ultracentrifugal data of the major low-density component (and assuming spheres), Oncley (6) obtained a molecular weight of 2.3 million and a hydrated density of 1.032 g/ml. The most

TABLE III

Correlations of S_f° Rate, σ and Molecular Weight, with S_f 0-20 Lipid Composition, Normals[a]

Parameter	PL	CE	TG	σ (lipid)
S_f°	0.79[b] (0.27)	-0.02 (0.07)	-0.61[c] (-0.30)	0.47 (0.26)
σ (lipoprotein)	-0.70[b] (-0.29)	-0.04 (0.03)	0.58[c] (0.23)	-0.47 (-0.17)
Mol wt	0.70[b] (0.23)	-0.11 (0.21)	-0.46 (-0.36)	0.34 (0.36)

[a]Normal females and males (in parentheses).

[b]$p < 0.01$.

[c]$p < 0.05$.

TABLE IV

Results, S_f 0-12 Major Component, Kaiser Clinical Referrals, Fasting

Age (mean ± SD)	Males (n = 16) 49 ± 11 years	Females (n = 19) 51 ± 10 years	Difference
1. S_f°	5.85 ± 1.41	6.74 ± 0.97	$p < 0.05$
2. σ (lipoprotein)	1.0327 ± 0.0053	1.0293 ± 0.0037	$p < 0.05$
3. Mol wt (millions)	2.16 ± 0.28	2.29 ± 0.0023	NS
Correlations			
1. S_f° vs σ	-0.96[a]	-0.90[a]	
2. S_f° vs mol wt	0.89[a]	0.72[a]	
3. σ vs mol wt	-0.73[a]	-0.35	

[a]Significance, $p < 0.01$.

recent hydrodynamic lipoprotein data is that of Adams (3) and Adams and Schumaker (8,19). Adams (3) utilized equilibrium banding in the analytic ultracentrifuge, patterned after Meselson (20), to obtain lipoprotein hydrated densities. From the principal σ component resolved and a single flotation rate measurement in a very high-density medium of sodium bromide (ρ = 1.481 g/ml) he obtained minimum hydrated molecular weights in the range of 1.94-2.38 millions. In a later study (8), Adams' earlier data were reconsidered using a frictional factor (including shape and hydration) f/f_0 = 1.2, giving corresponding higher molecular weight values in the range of 2.43 - 2.98 millions. Adams and Schumaker's most recent study (19) included a revision in technique with the assumption of lipoprotein density σ = 1.030 g/ml and a single high salt flotation measurement in a medium of ρ = 1.412 g/ml NaBr. Here, both a shape factor of f/f_0 = 1.05 and a hydration of 10% was assumed. The mean and standard deviation for nine LDL samples (fasting males, ages 20-40) was 2.38 ± 0.13 millions. Had they assumed spheres and no hydration the value would have been approximately 2.16 ± 0.13 millions. Our data on both normals and clinical referrals are approximately of this magnitude. Recently, from equilibrium data, Scanu et al. (21) have given a range of 2.2-2.3 million molecular weight units for the major low-density component, $1.019 < \sigma < 1.063$ g/ml.

Although from theoretical considerations values obtained by our technique might be expected to be low by as much as 10%, the differences between males and females as well as the relationship observed between S_f° rate, σ and molecular weight would appear to be valid. The results, of course, would apply to the small populations studied. However, it would seem unlikely, for example, that the shape factor for S_f° 4-8 lipoproteins would be different in males and females or would vary significantly from, say, S_f° 4 to S_f° 8. In spite of difficulties in applying corrections for concentration dependence in the flotation measurements, there is no evidence that any of the observed relationships and relative differences are K factor dependent. Recalculating all data using K factors of 0.44 or 1.78 x 10^{-4} $(mg/100\ ml)^{-1}$ did not significantly alter any of the relationships observed. It is estimated that a 20% error in the K factor used here, 0.89 x 10^{-4} $(mg/100\ ml)^{-1}$, would involve approximately a 5% error in molecular weight. However, as more concentration dependence data and shape factors become available, we may anticipate even more meaningful and accurate molecular weight data using this technique.

ACKNOWLEDGMENTS

E. H. Strisower, Kaiser Hospital, Oakland, Calif., made available the series of clinical referral samples. This work was supported, in part by Research Grant 5-RO1-HE-01882-14 from the National Heart Institute, U.S. Public Health Service, Bethesda, Md. and by the U.S. Atomic Energy Commission.

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[Received June 3, 1968]

The Effect of Dietary Copper on the Structure and Physical Properties of Adipose Tissue Triglycerides in Pigs

W. W. CHRISTIE and **J. H. MOORE**,
Hannah Dairy Research Institute, Ayr, Scotland

ABSTRACT

The inner back fat of control pigs had a higher melting point and stearic acid-oleic acid ratio than did the outer back fat of the control animals or the inner and outer back fats of pigs given the control diet supplemented with 250 ppm copper. By a combination of argentation thin layer chromatography and pancreatic lipase hydrolysis, it was shown that in the inner back fat of the control animals the proportions of two of the more saturated glyceride species were significantly greater and the proportions of two of the more unsaturated species were less than in the outer back fat of the control animals and in the inner and outer back fats of the copper-fed animals. These findings probably account for the observed differences in melting point. Stereospecific analyses of the glycerides demonstrated that the increased content of stearic acid in the inner back fat of the control pigs was distributed between the 1 and 3 positions.

INTRODUCTION

Copper, at a level of 250 ppm, is now routinely incorporated into the diets of pigs as it increases their growth rate and improves their food utilization efficiency. The topic has been reviewed by Hays (1) and Braude (2).

In a recent paper (3), we described the effect of incorporating 250 ppm copper in the diets of pigs on the fatty acid composition and physical properties of their adipose tissue. Pig back fat consists of two physically distinct layers of fat separated by connective tissue. The melting point of the inner back fat of the control animals was 10-15 C higher than that of the outer back fat of the control pigs or those of the inner and outer back fats of the pigs given the copper-supplemented diet. At the same time the stearic acid-oleic acid ratio in the inner back fat of the control animals was higher than that in the outer back fat of the control pigs or in the inner and outer back fats of the copper-fed pigs. As interesterification with sodium methoxide brought each fat to approximately the same melting point, it was evident that there must also be differences in glyceride structure. Accordingly, we now report structural analyses of the triglycerides from the inner and outer back fats of control and copper-fed animals.

EXPERIMENTAL PROCEDURES

The back fats from four control and four copper-fed pigs from experiments 4 and 5 in the previous paper in this series (3) were used in this study. The pigs (Large Whites) were given the control diet or the control diet supplemented with 250 ppm copper (1 g $CuSO_4.5H_2O$/kg of feed) from weaning until they reached 90 kg live weight when slaughtered. Strips of back fat, about 2 cm in width, were taken from each pig and the inner and outer layers separated along the line of connective tissue. The methods for the extraction of tissues and determination of melting points were described earlier (3).

Fatty Acid Analysis

Lipid samples were converted to the methyl esters of the constituent fatty acids with sodium methoxide in anhydrous methanol; when necessary benzene was added to facilitate solution. Gas chromatographic analyses were carried out on columns of 15% (w/w) polyethyleneglycoladipate on 100-120 mesh Chromosorb W, acid-washed and silanized (Phase Separations Ltd., Rock Ferry, Cheshire), in a Pye 104 chromatograph (Pye Unicam Ltd., Cambridge). Results were converted to moles per cent by multiplying the detector responses by the appropriate factors.

Silver Nitrate Thin Layer Chromatography

Triglyceride samples (7-10 mg) were applied in a band on plates (20 cm^2) coated with 0.5 mm thick Kieselgel G (E. Merck, Darmstadt) containing 10% (w/w) silver nitrate. The plates were given a double development at 4 C in a solvent system of toluene-diethyl ether (95:5 v/v). These conditions are similar to those of Morris et al. (4) for the separation of unsaturated methyl esters. After the plates had been sprayed with 2,4-dichlorofluorescein in methanol (0.1% w/v), the bands were visualized under UV light and scraped off with a razor blade into a small chromatography column containing 1 g Florisil (BDH Ltd., Poole, Dorset). The triglyceride fractions were eluted from the columns with diethyl ether, 100 ml. A solution (1 ml) of methyl heptadecanoate in methanol

(0.1261 g of ester in 250 ml of methanol) was added as an internal standard to each fraction, which was then transesterified before analysis by GLC. The weight of each fraction was calculated by reference to the amount of internal standard (5).

Analyses were accepted when they passed two tests: first, that the fatty acid composition of each individual fraction was within 1 unit per cent of the theoretical proportion of saturated, monoenoic or dienoic acids, and second, the value for the percentage of each fatty acid, calculated from the glyceride composition of the sample and the fatty acid composition of each glyceride fraction, was within 1 unit per cent of the corresponding value determined directly on the unfractionated triglyceride sample.

Pancreatic Lipase Hydrolysis

Triglyceride (200 mg) was applied to eight silver nitrate-impregnated TLC plates which were developed as above. Appropriate fractions from each were combined. Pancreatic lipase hydrolysis was carried out on 5-10 mg samples of each fraction by the procedure of Luddy et al. (6). With the more saturated fractions hexane (0.25 ml) was added to the lipolysis medium to ensure a more homogenous dispersion.

Stereospecific Analysis of Triglycerides

Brockerhoff's (7) first method of stereospecific triglyceride analysis was adopted with the following practical modifications.

The triglyceride (1 g) was dissolved in (4 ml) hexane. To this was added tris buffer (15 ml; 1 M, pH 7.5), calcium chloride solution (1 ml, 20% w/v) and sodium taurocholate solution (4 ml, 0.1% w/v). The whole was equilibrated at 38 C, then 0.4 g pancreatic lipase (Sigma Chemical Co., St. Louis, Mo.) was added and the mixture shaken vigorously in a mechanical shaker. After about 4 min 50% hydrolysis was achieved. Ethanol (10 ml) was added to stop the reaction; the reaction mixture was poured into 0.1 N HCl and the lipids were extracted with diethyl ether. The diglycerides were obtained by an initial separation on a silicic acid column (8) followed by preparative TLC on Kieselgel G plates (0.5 mm thick) impregnated with 5% (w/w) boric acid; the solvent system was hexane-diethyl ether (50:50 v/v). A small portion was esterified to check that random diglycerides had indeed been produced.

Phospholipid (~100 mg), prepared by Brockerhoff's procedure (7), was dissolved in diethyl ether (5 ml), then 0.5 M triethylammonium bicarbonate solution (3 ml, pH 7.5), 2% calcium chloride solution (0.05 ml) and 10 mg *Crotalus adamanteus* snake venom (Sigma Chemical Co., Ltd., St. Louis, Mo.) were added. The mixture was shaken vigorously overnight. Two drops of acetic acid and 20 ml isobutanol (to prevent foaming) were then added and the mixture was taken to dryness. The lipid mixture was dissolved in a little chloroform-methanol (2:1 v/v) and applied as a band to a Kieselgel G TLC plate (0.5 mm thick), which was then developed with hexane-ether-acetic acid (50:50:1 v/v). With this solvent system the unchanged phosphatide and lysophosphatide remained near the origin and were well separated from the free fatty acids. The phosphatides were detected by spraying with methanolic dichlorofluorescein and the appropriate band scraped off into a small chromatography column and eluted with 100 ml chloroform-methanol (2:1). The phosphatides were then reapplied to a Kieselgel G plate (0.5 mm thick) which was developed with chloroform-methanol-ammonia, (85:15:2 w/v). With this solvent system, the diacyl phosphatide has an R_f value of ~0.4 and the lysophosphatide an R_f value of ~0.2. The phosphatide bands were detected by spraying with aqueous Rhodamine 6 G (0.01% w/v), recovered from the plate as before and transesterified for fatty acid analysis by gas liquid chromatography (GLC).

The fatty acid composition of the lysophos-

TABLE I

Fatty Acid Compositions (Mole Percentages of the Total) and Melting Points of Inner and Outer Back Fats From Control and Copper-fed Pigs

Diet	No. of pigs	Type of back fat	Fatty acid composition						Melting point (C)
			14:0	16:0	16:1	18:0	18:1	18:2	
Control	4	Inner	1.8±0.1	28.8±0.4	2.5±0.2	18.5±0.6	39.5±1.6	8.9±1.1	45+1
Copper-supplemented	4	Inner	2.0±0.1	28.7±1.1	3.2±0.6	15.7±1.7	42.4±2.0	8.0±1.1	34+1
Control	4	Outer	1.9±0.4	28.0±1.2	3.0±0.3	15.2±1.0	41.6±1.6	10.3±1.1	31+3
Copper-supplemented	4	Outer	2.0±0.1	27.7±0.5	3.8±0.7	12.9±1.6	44.7±1.8	8.9±0.4	33+2

TABLE II

Triglyceride Types (Mole Percentages of the Total) in the Inner and Outer Back Fats from Control and Copper-fed Pigs

Diet	No. of pigs	Type of back fat	Triglyceride type						
			S_3	S_2O	SO_2	O_3	S_2L	SOL	Rest
Control	4	Inner	7.0±0.7	33.0±1.6	29.0±2.5	5.3±0.6	7.4±0.8	9.5±2.3	8.8±1.0
Copper-supplemented	4	Inner	5.3±1.1	29.8±2.9	31.7±3.8	6.5±1.4	6.7±1.2	10.7±1.3	9.3±1.8
Control	4	Outer	4.9±0.7	27.1±1.2	30.8±1.8	6.6±0.7	6.8±0.8	12.3±1.1	11.5±0.7
Copper-supplemented	4	Outer	4.2±1.0	27.1±2.8	33.8±1.8	8.1±1.7	5.6±1.0	11.5±0.8	9.7±1.2

phatide is that of the 1 position of the triglyceride and the composition of the 2 position can be obtained from the results of degradation studies with pancreatic lipase. The composition of the fatty acids in the 3 position can be calculated either from the results for the unhydrolyzed triglyceride and the results for the 1 and 2 positions or from the results for the 2 position and the results for the unchanged phosphatide. In accordance with the suggestions of Brockerhoff et al. (9), analyses were accepted when the values for the fatty acid composition of the 3 position calculated by the two possible methods agreed within 4% for major components to 2% for minor components.

RESULTS AND DISCUSSION

The fatty acid compositions and melting points of the inner and outer back fats of the control and copper-fed pigs are detailed in Table I. Only six fatty acids, myristic, palmitic, stearic, palmitoleic, oleic and linoleic acids were present in appreciable concentrations (< 0.5% of others). The inner back fat of the control animals had a higher melting point and a higher stearic acid-oleic acid ratio than did the outer back fat of the control pigs or the inner and outer back fats of the copper-fed animals. Similar results have been discussed in detail previously (3).

Thin layer chromatographic (TLC) analysis confirmed that triglycerides were the only lipid class present. By silver nitrate TLC under the conditions described, five fractions, differing in the degree of unsaturation, were clearly separated and identified by GLC analysis as S_3, S_2O, SO_2, O_3 + S_2L and SOL (using the notation S for saturated, O for monoenoic and L for dienoic fatty acids). The remaining more unsaturated species could be separated into a large number of bands by a more polar solvent system but as these constituted only a small proportion of the whole, it was believed unnecessary to investigate these species in detail. The fourth fraction, which was a mixture of O_3 and S_2L could occasionally be separated into its two components although there was usually some overlap. However, the relative amounts of each component could be established from the fatty acid composition.

TABLE III

Triglyceride Types (Mole Percentages of the Total) From the Inner and Outer Back Fats of Individual Control and Copper-fed Pigs

Glyceride type	Tissue			
	Control inner	Copper-fed inner	Control outer	Copper-fed outer
SSS	6.3	4.2	4.4	3.1
SSO	32.2	25.9	26.8	22.8
SOS	0.7	0.7	0.5	0.9
SOO	5.2	5.5	5.0	5.6
OSO	25.5	29.7	28.1	29.9
OOO	5.0	7.6	6.6	10.4
SSL	6.5	5.3	5.9	4.6
SLS	0.2	0.3	0.3	---
SOL	1.2	1.5	1.6	1.4
SLO	0.9	0.9	1.1	1.0
OSL	7.7	7.9	9.1	9.1
Rest	8.6	10.5	10.6	11.2

TABLE IV

Fatty Acid Distribution (Mole Percentages) in the Triglycerides of the Inner and Outer Back Fats of Individual Control and Copper-fed Pigs

Diet and tissue	Pig No.	Position	Fatty acid composition					
			14:0	16:0	16:1	18:0	18:1	18:2
Control inner	1	1	1	9	2	33	47	8
		2	4	74	4	4	11	3
		3	--	3	2	17	66	12
Control inner	2	1	1	9	2	34	46	8
		2	4	74	4	4	11	3
		3	1	5	2	17	62	13
Control outer	1	1	1	9	2	27	52	9
		2	5	71	4	3	13	4
		3	2	9	3	11	62	13
Control outer	2	1	1	9	2	29	50	9
		2	4	70	4	4	13	5
		3	1	3	2	16	63	15
Copper inner	3	1	1	14	3	26	48	8
		2	5	70	6	3	13	3
		3	--	--	3	11	73	13
Copper inner	4	1	1	11	3	26	51	8
		2	5	74	4	3	11	3
		3	--	6	3	17	66	8
Copper outer	3	1	1	10	3	21	56	10
		2	5	66	7	3	15	4
		3	--	5	4	10	69	12
Copper outer	4	1	1	9	3	24	55	8
		2	5	73	5	3	11	3
		3	--	2	3	13	68	14

The proportion of each triglyceride species from the inner and outer back fats of the control and copper-fed pigs are given in Table II.

The proportions of trisaturated and disaturated-monounsaturated species in the triglycerides of the inner back fat of the control pigs were much higher than the corresponding proportions in the triglycerides of the outer back fat of the control animals or the inner and outer back fats of the copper-fed animals. At the same time, the proportions of the species SO_2 and O_3 were lower in the inner back fat of the control pigs than in the triglycerides from the other three types of adipose tissues.

By preparative TLC on a number of silver nitrate-impregnated plates it was possible to isolate sufficient of each triglyceride class for degradation studies with pancreatic lipase hydrolysis. This procedure permitted a more detailed subdivision of triglyceride species and has been used previously by Blank et al. (10) to examine lard. The results obtained by this technique on the inner and outer back fats from a control pig and a copper-fed pig are given in Table III. From these results it is evident that the major change in the triglyceride type S_2O is in the SSO fraction rather than the SOS, and that in the SO_2 fraction the species OSO changes rather than the SOO.

As natural fats are such complex mixtures of triglyceride species, very little is known about the relationship between physical properties and triglyceride types, particularly as polymorphism within individual triglycerides can be a complicating factor. The subject has been reviewed by Rossell (11). However, it is generally assumed that the proportions of the solid triglycerides (S_3 and S_2O) largely govern the physical properties, in particular the melting and softening points of a fat. Therefore, although it is impossible to predict or comment on the magnitude of the effect, it is not surprising that, in view of its higher content of the more saturated triglyceride species, the inner back fat of control pigs has a higher melting point than the other samples of back fats. Most of the changes in fatty acid composition with dietary treatment were small but were reflected in the proportions of only two or three of the many triglyceride classes. It is theoretically possible, therefore, for a small increase in the total stearic acid concentration of a fat, for example, to result in a comparatively large increase in the concentration of the single species SSO. Thus, a small change in the fatty acid composition of pig adipose tissue can have an apparently disproportionate effect on the physical properties of the fat.

Recently, Brockerhoff (7,12) and Lands et al. (13) have described methods for the stereospecific analysis of triglycerides, i.e., for determining the composition of the fatty acids

esterified in positions 1, 2 and 3 of L-glycerol. Lard (9) was shown to have a rather unusual distribution of fatty acids with 80% of the palmitic acid in position 2 and 70% of the remaining saturated fatty acids in position 1. It therefore seemed of interest to compare the stereospecific distribution of the fatty acids in the triglycerides from the inner and outer back fats of the control and copper-fed animals to investigate whether the small changes in fatty acid composition of the whole triglyceride are reflected in greater changes in any of the positions in the glycerides. Brockerhoff (12) states that "lard is an exceptionally difficult fat to analyze" and recommends the first of his procedures. This was adopted in preference to that of Lands et al. (13) as the necessary enzymes for the purpose were more readily available.

Brockerhoff's method (7) involves the preparation of random diglycerides by hydrolysis with pancreatic lipase, the preparation of a synthetic phospholipid from the resulting diglycerides and finally the hydrolysis of this phospholipid with the stereospecific phospholipase A of snake venom. As only the L-phosphatide is hydrolyzed by this enzyme, the fatty acid composition of the resulting lysophosphatide is that of the 1 position. The composition of the 2 position can be determined by pancreatic lipase hydrolysis of the original triglyceride and that of the 3 position then calculated by difference. The fatty acid composition of the 3 position can also be calculated from that of the unchanged D-phosphatide, so a check on the results is possible. In our hands the method gave reproducible results after minor practical changes in the preparation of the diglycerides by pancreatic lipase hydrolysis and in the phospholipase A hydrolysis and subsequent isolation of products. Analyses were performed on the inner and outer back fats from two control and two copper-fed animals and the results are listed in Table IV.

Our results are similar to those of Brockerhoff, et al. (9) for lard made from commercially salted pork. The fatty acid compositions in the 2 position of the triglycerides of the inner and outer back fats of the control and copper-fed pigs did not differ markedly and were 70% palmitic acid. The stearic acid content was higher in both the 1 and 3 positions in the inner back fat of the control pigs than in the outer back fat of the controls or the inner and outer back fats of the copper-fed animals; the increase in stearic acid content occurred largely at the expense of oleic acid. Apparently, therefore, the effect of the dietary copper on the fatty acid composition of the triglycerides of the adipose tissue of the pig is not exerted preferentially in any single position of the glycerides.

It is not known by what mechanism dietary copper affects lipid metabolism in the pig. Taylor and Thomke (14) have speculated that dietary copper might affect fat absorption or transport or that the high level of copper found in the liver of animals on copper-supplemented diets might affect the normal metabolism of this tissue. It has been suggested that the temperature at the site of fat deposition determines fatty acid composition in the pig (15) and some experimental evidence (16) supports this theory. Similarly, we suspect that dietary copper may affect the lipid metabolism of the pig only indirectly. Further work will be necessary to confirm this hypothesis.

ACKNOWLEDGMENTS

Technical assistance by Miss J. Begg.

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[Received October 4, 1968]

Studies on the Lipids of Sheep Red Blood Cells: III. The Fatty Acid Composition of Phospholipids in HK and LK Cells

GARY J. NELSON Bio-Medical Division, Lawrence Radiation Laboratory, University of California, Livermore, California 94550

ABSTRACT

The fatty acid composition of the erythrocyte phospholipids was studied in samples from five high potassium (HK) and five low potassium (LK) sheep. The total fatty acid composition, including the composition from the individual phospholipids in the erythrocytes of these animals is reported. There were no significant differences between either the total fatty acid composition or that of the individual phospholipids in the HK or LK cells. Sheep red cells had very little polyunsaturated fatty acids in their phospholipids. Palmitic, stearic and oleic acids were the major components of glyceryl phospholipids, while nervonic acid accounted for 50% of the fatty acids in the sphingomyelin fraction. The similarity between the fatty acid composition of HK and LK red cells indicates that quantitative differences in the lipids of the membrane are not the primary reason for the observed differences in the cation levels in the two types of cells. This agrees with conclusions drawn from previous studies.

INTRODUCTION

Previous studies on the fatty acid composition of the mammalian red blood cell were usually limited to the total fatty acids present without regard to fatty acid composition of the individual phospholipid in the cell membrane (1-4). Data from human (5-8) and rat (9-12) red cells are more extensive than for other species. However, since studies on the phospholipid distribution in erythrocytes and plasma of other species (13-16) have shown marked species variability, it is very likely that the fatty acid composition of the individual phospholipids in other species is quite different from that in human or rat. Therefore, further studies in this area may help to clarify the problem of the structure of the erythrocyte membrane.

Earlier studies in this laboratory on the lipids of HK (high potassium) and LK (low potassium) sheep (16,17) established that there is no difference in the distribution of the individual lipid classes between the HK and LK cells. Thus, it is unlikely that the differences in the cation transport processes can be accounted for by differences in the lipid composition of the membrane. The possibility remained, however, that the permeability of the membrane is affected by the fatty acid composition of the constituent lipids, i.e., the liquid crystalline state of the membrane (18). This report gives the result of an investigation of the composition of the total red cell fatty acids and that of the individual phospholipids in samples from five HK and five LK sheep.

MATERIALS AND EQUIPMENT

A flock of purebred Hampshire sheep is maintained at this laboratory and specified as HK or LK on the basis of blood analysis by flame spectrophotometry. The diet and the husbandry of these animals have been described earlier (16,17).

Standard phospholipid preparations were obtained from Applied Science Laboratories, State College, Pa. and General Biochemicals, Chagrin Falls, Ohio. Pure fatty acid methyl esters were obtained from Applied Science Laboratories. All solvents were redistilled from glass stills and deoxygenated with N_2 before use. BHT (2,6-di-*tert*-butyl-*p*-cresol) was used as an antioxidant in all operations and was added to the solvents and samples in all phases of the experimental procedure.

Thin layer chromatography (TLC) was carried out with equipment obtained from Brinkman Instruments, Inc., Westbury, N.J. Optical densities for the phosphorous determinations were measured with a Zeiss PMQ II spectrophotometer. Gas liquid chromatography (GLC) was carried out on a Barber-Colman Model-10 C gas chromatograph equipped with a ^{90}Sr Argon detector. The data obtained from the gas chromatograph were analyzed by a computer technique (19,20) using a Control Data 3600 digital computer.

EXPERIMENTAL PROCEDURE

Sampling and Isolation of Red Cells

Whole fresh blood was drawn and immediately cooled to 0 C and thoroughly washed to

remove plasma and leucocytes by the methods described in detail previously (13,15), using heparin to prevent clotting. The leucocyte count was below one cell per 10^4 erythrocytes using Giesma stained preparations.

Chromatographic Methods

The washed, whole-cell preparations were extracted by the methods already described (13,15), and the extracts were purified by column chromatography on Sephadex using the procedure of Siakotos and Rouser (21) as modified slightly (13,22). Fractions 2 and 3 from the Sephadex column were not processed further.

The total lipid extract (Fraction 1 from the Sephadex column) was dissolved in $CHCl_3$-MeOH (19:1 v/v) and stored at -10 C until further analysis. Aliquots were taken immediately for phosphorous, total weight and total fatty acid analyses; samples were stored in the presence of BHT.

The two-dimensional TLC separations of the phospholipids in the total lipid extract were performed by methods developed by Rouser et al. (23,24). Silica Gel HR mixed with 10% $MgSiO_2$ by weight was the TLC adsorbent. Details of the procedures, including plate preparation techniques and development solvents, have been described elsewhere (13,14,23,24). Quantitative determinations of the phospholipids were carried out by spectrophotometric procedures on spots scraped from the charred TLC plates without removing the adsorbent (13,25).

The procedure for separating the phospholipids for the analysis of their component fatty acids was essentially identical with the previously reported methods up to the time the plates were removed from the second developing solvent. At this time the solvent was removed by placing the plate briefly in a dry box under a stream of N_2. The plate was then removed from the chamber and sprayed lightly with 2′,7′-dichloroflourescein (dissolved in methanol, 2% by weight). The spots were then viewed under ultraviolet light (3200 A) in a viewing box (Ultraviolet Products, San Gabriel, Calif.) and outlined at this time. To obtain enough sample for analyses of some of the minor phospholipids, it was necessary to prepare plates for each sample in triplicate. The individual phospholipid spots were then scraped directly into transmethylation tubes. No attempt was made to remove the adsorbent or dye and the corresponding spots from the three plates were pooled in the same transmethylation tube.

Transmethylation Procedure

Dry HCl methanol, approximately 7% by weight HCl, was prepared after the procedure described by Farquhar et al. (26). Five milliliters of HCl methanol was added to each transmethylation tube and the tubes were capped with a reflux condenser and drying tube. The tubes were then heated for 2 hr at approximately 90 C. Boiling chips were added to each transmethylation tube to prevent the violent bumping that occurred because of the adsorbent in the tubes; when no adsorbent was present, no bumping occurred even without boiling chips. The boiling chips were, of course, washed thoroughly with acid and organic solvent before use.

The tubes were then cooled to room temperature and 10 ml of ice-cold distilled water was added to each tube. The methyl esters were then extracted with three washes of 3 ml of hexane. After the hexane was removed, the samples were transferred to tared, 2 ml vials and an approximate weight was obtained to facilitate proper dilution for injection into the gas chromatograph.

For samples that contained material other than fatty acid methyl esters, the sample was rechromatographed on a small column (15) of silica gel. Usually when this procedure was followed, two fractions were taken: the first, 1% diethyl ether in hexane, contained the methyl esters and BHT; the second, pure methanol, contained any additional hexane-soluble material from the transmethylation. The composition of the fractions from the small columns was checked by TLC using hexane-diethyl ether-acetic acid (80:20:2 v/v) as the solvent system (15). This latter material could be cholesterol (in the case of the total lipid extract), lysophosphatidyl ethanolamine glyceryl ether or sphingosine, depending on which particular sample was being transmethylated. Sheep erythrocytes contain no vinyl ether phospholipids and therefore do not yield dimethyl acetals upon transmethylation (G. Nelson, unpublished observation). Also, fortunately, as reported by Dodge and Phillips (8), the dichloroflourescein remains entirely in the aqueous phase of the transmethylation and does not interfere with subsequent analyses of the methyl esters.

Gas Liquid Chromatography

The GLC was performed on the samples dissolved in hexane. The stationary phase used for the quantitative analysis was 15% diethylene glycol succinate (HI-EFF-1BP, Applied Science Laboratories, Inc.) on 60-80 mesh Gas-Chrom P. Fifteen per cent Apiezon L

TABLE I

Distribution of Major Fatty Acids[a] in HK and LK Sheep Erythrocytes

Fatty acid	Per Cent of Total Cellular Fatty Acids (as Methyl Esters)[b] LK n = 5	HK n = 5
16:0	8.7 ± 2.9 (4.0 - 10.0)	10.2 ± 0.4 (9.5 - 10.9)
16:1	0.6 ± 0.1 (0.5 - 0.8)	0.8 ± 0.1 (0.7 - 0.9)
17:0	0.9 ± 0.2 (0.6 - 1.3)	1.3 ± 0.1 (1.1 - 1.4)
17:1	0.5 ± 0.1 (0.4 - 0.6)	0.7 ± 0.1 (0.6 - 0.9)
18:0	8.3 ± 0.9 (7.1 - 9.7)	8.4 ± 0.7 (7.8 - 9.7)
18:1	40.3 ± 3.3 (34.4 - 43.4)	38.8 ± 1.8 (35.9 - 40.9)
18:2	8.5 ± 0.9 (6.9 - 9.4)	8.3 ± 1.1 (7.6 - 10.3)
20:0	1.2 ± 0.1 (1.1 - 1.3)	1.0 ± 0.2 (0.8 - 1.3)
22:0	1.8 ± 0.3 (0.9 - 2.0)	2.0 ± 0.4 (1.4 - 2.4)
24:0	2.0 ± 0.4 (1.6 - 2.7)	1.9 ± 0.2 (1.7 - 2.1)
24:1	16.3 ± 2.6 (13.2 - 19.4)	14.6 ± 1.6 (12.5 - 16.8)
25:0	1.8 ± 0.2 (1.5 - 2.1)	2.4 ± 0.5 (1.8 - 3.5)
Sum of minor fatty acids	9.1 ± 1.8 (6.8 - 11.4)	9.7 ± 1.6 (5.8 - 10.8)

[a] Only including those that contribute more than 0.5% of the total fatty acids.

[b] Average values ± standard deviations; ranges are in parentheses.

was also used to identify the constituent fatty acids, but only qualitatively. Standard methyl ester mixtures obtained from Applied Science Laboratories and corresponding to NIH mixtures KA, KB and KD, as well as Applied Science Laboratories Quantitative Mixtures H-104, K-105 and K-107, were used to determine the quantitative accuracy of the fatty acid analysis. The computer program used the product of peak height (arbitrary units) and absolute retention time (in minutes after injection) to calculate the relative areas of each peak with a number of empirical correction factors applied to the measured peak heights to compensate for detector nonlinearity and nonuniform response of individual methyl esters. On our instrument, the relationship between retention time and triangulated base width was linear within the error of measurement for all standard methyl esters that could be obtained from commercial sources, provided that the column was operated isothermally. All quantitative data were taken from isothermal GLC runs.

The quantitative results calculated by the computer program for the known methyl ester samples agreed within 10% of the stated composition for components present in amounts greater than 10% of the total sample and 20% for components less than 10%. Repetitive analyses of the standard mixture agreed within 3% for all components regardless of the relative amount in the sample. Repetitive analyses of sheep erythrocyte fatty acids agreed within 5% in most cases.

The fatty acids were identified by the separation-factors method of Ackman et al. (27-29), as well as by comparison with the standard methyl ester's retention time on both diethylene glycol succinate and Apiezon L columns. However, because capillary columns were unavailable, positional isomers could not be distinguished in these analyses; therefore only the length of carbon chain and the total number of double bonds on the chain were used to designate the fatty acid methyl esters.

In addition, the BHT was not removed prior to sample injection into the gas chromatograph. As noted by Dodge and Phillips (8), this compound elutes from polar GLC phases with a retention time close to methyl myristate; therefore no values for myristic acid were obtained in this study. In a few samples in which no BHT was used, the amount of myristate acid was less than 0.5% of the total fatty acids so that this omission was not believed to impair the data to any serious degree.

Sample blanks were also prepared and carried through the entire analytical scheme. The blank readings were entered into the computer program and automatically subtracted from the samples. The major peak in the blank runs, besides BHT, had a retention time identical to that of methyl palmitate and presumedly was methyl palmitate, which is apparently ubiquitously distributed in the environment either as the free acid or methyl ester.

TABLE II

Distribution of the Major Fatty Acids in the Individual Phospholipids of HK and LK Sheep Erythrocytes (Per Cent of Total Fatty Acid Methyl Esters)[a]

Fatty acid	Unknown I			Phosphatidyl serine			Phosphatidyl ethanolamine			Phosphatidyl inositol		
	LK	n = 5	HK	LK	n = 5	HK	LK	n = 5	HK	LK	n = 5	HK
16:0	24.2 ± 2.1		26.2 ± 1.7	6.4 ± 0.2		6.7 ± 0.8	2.1 ± 0.6		2.5 ± 0.5	9.6 ± 0.3		9.1 ± 1.1
18:0	12.0 ± 2.3		9.8 ± 2.2	19.3 ± 1.1		19.4 ± 1.2	1.1 ± 0.2		0.8 ± 0.4	28.7 ± 1.7		28.6 ± 2.5
18:1	43.2 ± 6.0		42.3 ± 1.7	49.6 ± 2.7		49.2 ± 3.5	79.5 ± 0.7		78.3 ± 1.3	33.0 ± 1.8		31.7 ± 1.9
18:2	13.0 ± 2.8		11.3 ± 2.5	17.1 ± 2.1		15.2 ± 1.3	8.8 ± 1.4		8.9 ± 1.1	12.5 ± 2.4		10.8 ± 1.4
20:0	1.1 ± 0.2		0.9 ± 0.3	1.5 ± 0.2		1.1 ± 0.2	1.6 ± 0.2		1.3 ± 0.3	2.7 ± 0.1		2.5 ± 0.5
22:0	1.6 ± 0.2		1.7 ± 0.2	1.2 ± 0.1		1.7 ± 0.1	1.1 ± 0.1		1.6 ± 0.3	2.5 ± 0.5		3.2 ± 0.2
Sum of minor fatty acids	5.0 ± 1.8		7.9 ± 2.5	4.9 ± 2.0		6.8 ± 2.4	5.9 ± 1.0		6.7 ± 0.9	11.0 ± 3.7		14.2 ± 4.6

[a]Average values ± standard deviations.

RESULTS

There is essentially no difference in phospholipid composition between the two types of cells or between recent results and those obtained in the previous series (17). Table I gives the distribution of the fatty acids in the whole cell as per cent of total methyl esters for samples from both HK and LK animals. The fatty acid pattern of the sheep erythrocyte is characterized by the absence of polyunsaturated fatty acids. Linoleic acid is the only fatty acid containing more than one double bond present in sheep erythrocytes in amounts greater than 5% of the total fatty acids and it accounts for only 8% of the total fatty acids. This is in striking contrast to the value of 30% polyunsaturated fatty acids (6,8) reported for human erythrocytes.

There is no discernible difference in total fatty acid distribution between the HK and LK cells; average values, standard deviations and range are essentially the same. Tables II and III present the fatty acid distribution for the individual phospholipids in the erythrocytes of HK and LK animals. Sheep erythrocytes contain no lecithin or lysolecithin (16,17). Also, the phosphatidic acid level in these samples was too low for fatty acid analyses, as was the amount of Unknown II. The values for phosphatidyl ethanolamine, characterized by large amounts of oleic acid, are for the 2 position of the glyceryl ether only. Sheep red cells contain only the saturated ether analogue of phosphatidyl ethanolamine; none of the diacyl or vinyl ether compounds are present (G. Nelson, unpublished observation).

There is essentially no difference in the fatty acid distributions of the individual phospholipids between HK and LK cells. This was expected, of course, because the total fatty acid patterns showed no significant differences. However, a unique fatty acid pattern was associated with each phospholipid. The only similarity is found when phosphatidyl inositol is compared with phosphatidyl serine, and, even there, distinct differences are apparent. Tables I, II and III for each individual phospholipid list only the fatty acids present in amounts greater than 1% of the total fatty acids. Generally, only six fatty acids meet this criterion for the glycerol phospholipids; sphingomyelin is characterized by a large amount of nervonic acid. In most cases, however, the fatty acids present in excess of 1% account for 90% or more of the total fatty acids. Small differences between the average fatty acid distributions in the individual phospholipids of HK and LK cells are not considered significant and are attributed to

TABLE III

Distribution of the Major Fatty Acids in Sphingomyeline of HK and LK Sheep Erythrocytes

Fatty acid	Per Cent of Total Fatty Acid Methyl Esters[a] LK n = 5	HK n = 5
16:0	20.6 ± 0.8	18.4 ± 1.9
18:0	2.9 ± 1.0	3.1 ± 0.7
18:1	1.5 ± 0.5	2.3 ± 0.4
20:0	1.0 ± 0.3	1.1 ± 0.4
22:0	2.7 ± 0.4	2.9 ± 0.5
22:1	1.1 ± 0.5	1.3 ± 0.7
23:0	1.3 ± 0.3	1.4 ± 0.2
23:1	1.9 ± 0.3	1.8 ± 0.4
24:0	5.6 ± 1.2	5.4 ± 0.5
24:1	52.3 ± 2.8	52.3 ± 1.7
25:0	1.8 ± 0.3	1.8 ± 0.4
Sum of minor fatty acids	7.2 ± 0.8	8.4 ± 1.2

[a]Average values ± standard deviations.

experimental error. The ranges of values obtained in the HK and LK animals were essentially identical.

DISCUSSION

The data presented here tend to confirm the observation reported earlier (16) that HK and LK erythrocytes of sheep do not differ in the lipid composition of the membrane. A corollary to this observation is that the lipids of the erythrocyte membrane are not actively involved in cation transport across the cell membrane, or at least that the factors controlling transport are probably not located in the major membrane lipids. Certainly the lipid portion of the membrane, be it a bilayer or some other arrangement, is probably identical in HK and LK cells.

The fatty acid composition of sheep erythrocytes has not been studied extensively by other investigators. The total erythrocyte fatty acid composition was reported by Connellan and Masters (1), Kogl et al. (2) and de Gier and van Deenen (3). These investigators generally agreed that palmitic, stearic, oleic and linoleic acids were the major fatty acids present in sheep erythrocytes. It is curious, however, that none of these investigators reported the presence of nervonic or lignoceric acid in sheep erythrocytes. They did, however, report the presence of arachidonic acid, a substance that was not present in detectable amounts in the samples analyzed in this work.

The total fatty acid composition of human and rat erythrocytes has been studied most frequently (5-8,9-12). The distribution of the fatty acids in rat and human is similar and quite different from the pattern observed in sheep. Both rat and human erythrocytes contain considerable amounts of lecithin, but sheep erythrocytes contain none. This may explain some of the differences observed between sheep and the two other species. In addition, the phosphatidyl ethanolamine of human and rat erythrocytes is highly unsaturated (the diacyl or vinyl ether form), whereas in the sheep cell only the saturated ether form is present (G. Nelson, unpublished observation). It is doubtful that the differences between sheep erythrocytes fatty acids can be accounted for on the basis of different phospholipids. It is more likely that there is a basic difference in the fatty acids initially incorporated in the red cell membranes during erythropoiesis.

It is apparent from fatty acid analyses of erythrocytes of other species (8,12,30-32) that a wide range of fatty acids is used in the formation of the red cell membrane in a particular species. However, the analysis of erythrocyte fatty acids is complicated by fatty acid and phospholipid exchange phenomena in the circulation of most mammalian species. Oliveira and Vaughan (33) and Mulder et al. (34,35) have elucidated the pathway by which the 2 position fatty acid of lecithin can be exchanged with free fatty acids of plasma. Also, total exchange of intact red-cell phospholipids with plasma phospholipids can occur in man (36), dog (36), and rat (37), and presumably other species. These exchange reactions may well cause the erythrocyte fatty acid composition to reflect the composition of the dietary fat rather than that of newly synthesized red cells.

Sheep, on the other hand, may present a special case in which the fatty acid composition of the erythrocyte is relatively independent of the diet. The exchange reaction between lecithin and plasma free fatty acids cannot occur in sheep because the sheep cells have no lecithin. Reed (36) has shown that intact phospholipid can be exchanged between erythrocytes and plasma only if both cell and plasma contain appreciable quantities of that particular phospholipid. Since sheep plasma contains only insignificant amounts of phosphatidyl ethanolamine and phosphatidyl serine (14), only the erythrocyte sphingomyelin can be influenced by the exchange reaction, and that only slowly (36). Therefore, it is probable that the fatty acid composition of sheep erythrocytes is independent of the diet, except for de novo synthesis of new cells as demonstrated by van Deenen et al. (4).

There are only scattered and incomplete reports in the literature on the fatty acid

composition of the individual phospholipids of sheep erythrocytes. van Deenen et al. (4) reported values for three fatty acids of sheep cephalin (palmitic, oleic and arachidonic acids), and indicated that oleic acid was the major fatty acid in this group. This is in agreement with the findings reported here. There is, however, a larger body of data on the fatty acid composition of phospholipids from organs and tissues of sheep (38-41). Scott et al. (38) reported that palmitic, stearic and oleic acids were the major fatty acids associated with phospholipids from heart, liver, kidney and brain; similar results were found by Miller and Rice (39) for lamb liver, serum and muscle. Shorland et al. (41) reported findings similar to those of Scott et al. (38) and Miller and Rice (39) and, in addition, separated the phosphatidyl ethanolamine and lecithin from the whole bodies of fetal and maternal sheep and found again that palmitic, stearic and oleic acids were the major fatty acids in these phospholipids in both samples.

ACKNOWLEDGMENTS

Technical assistance given by R. A. Booth. This work was performed under the auspices of the U.S. Atomic Energy Commission.

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[Received October 9, 1968]

Microbial Hydration of *Cis*-9-Alkenoic Acids

E. N. DAVIS, L. L. WALLEN, J. C. GOODWIN, W. K. ROHWEDDER and **R. A. RHODES** Northern Regional Research Laboratory,[1] Peoria, Illinois 61604

ABSTRACT

The activity of a *cis*-9-fatty acid hydratase produced by a *Pseudomonas* sp. (NRRL B-3266) isolated from soil was compared with that of another isolate previously reported (NRRL B-2994). The presence of appropriate fatty acids for at least 4 hr during aerobic growth in yeast extract medium increased subsequent enzyme activity. Such cells anaerobically hydrated several *cis*-9-alkenoic acids to 10-hydroxy fatty acids and aerobically formed 10-keto acids, which were partially degraded to shorter chain keto acids. Melting point, gas chromatography, infrared, mass spectrometry and optical rotatory dispersion data are given. Six fatty acids having *cis*-9-unsaturation produced hydrated products, but several enoic acids having *trans*-9-unsaturation or double bonds in other than the 9 position were inactive as substrates. The (-)-10-hydroxypalmitic acid produced from palmitoleic acid is considered to have the D configuration. Yields of 71% crude crystalline product from 15 g of oleic acid and 53% from 11 g of palmitoleic acid were obtained in 5-liter anaerobic fermentations with NRRL B-3266. Methyl esters, triolein and oleyl alcohol were not hydrated.

INTRODUCTION

Ricinoleic acid from castor oil is the only hydroxy fatty acid commercially available. Orujo (sulfur olive) oil was reported to contain 10-hydroxystearic acid (1); however, the low melting point reported (101-103 C) for the semicarbazone of the keto derivative compared to the melting points for the semicarbazones of authentic 9-keto and 10-ketostearic acids (2) suggests that a mixture of 9- and 10- hydroxystearic acids was isolated from this oil. There is no example of a 10-hydroxy acid among recently studied natural fatty acids in seed lipids (3) although *Cardamine* seed oil contains 9,10-dihydroxystearic acid. Of the 628 literature citations to oxidations by microorganisms compiled by Wallen et al. (4) in 1959, there is no example of a long-chain unsaturated fatty acid being hydrated to a hydroxy acid although in one reaction cited (5) oleic acid is converted to either 9- or 10-ketostearic acid. The keto acid probably would have been formed by dehydrogenation from an intermediate 9- or 10-hydroxystearic acid.

In 1962, Wallen et al. (2) found that the unidentified *Pseudomonas* sp. NRRL B-2994 (ARS Culture Collection at the Northern Laboratory) (6) converted oleic acid to 10-hydroxystearic acid in about 14% yield. Schroepfer and Bloch (7,8) showed that this product was optically active ($[\alpha]_{546} = -0.16$) and that the 10-hydroxyl had the D configuration, whereas the hydrogen added at the 9 carbon had the L configuration. They found the rotation to be positive when the 10-hydroxyl was chemically transposed to the L configuration.

During work on modifying common seed oil fatty acids by microbial action, a different *Pseudomonas* sp. was isolated that appeared to cause the same reaction catalyzed by NRRL B-2994. We wish to report comparisons indicating identical enzyme activity by both organisms, together with studies on the specificity of the enzyme, by-product reactions with whole cell cultures and optimization of yields of 10-hydroxy fatty acids.

MATERIALS AND METHODS

Most fatty acids, together with their esters and alcohols, were obtained from The Hormel Institute and had greater than 99% purity. The purity of the Hormel triolein was more than 90%. Myristoleic acid was purchased from Applied Science Laboratories. *Cis*-5-eicosenoic acid (9) in excellent purity was the gift of F. G. Dollear, Southern Regional Research Laboratory.

Microorganisms were isolated from soil samples collected aseptically from a local meat-processing plant and from a soybean oil mill. The soils were plated on ammonium nitrate-salts agar containing fine droplets of a dispersed mixture of vegetable oil fatty acids. Well-separated surface colonies were picked and purified by further plating; the resultant pure cultures were maintained on slants of tryptone-

[1]No. Utiliz. Res. Dev. Div., ARS, USDA.

glucose-yeast extract (TGY) agar (6). Before use in fermentations, subcultures were transferred at least twice in a yeast extract (YE) medium incubated on a rotary shaker.

The YE medium used for growth and fermentation contained 0.25 g $MgSO_4$, 0.3 g K_2HPO_4, 0.7 g KH_2PO_4 and 5 g Difco yeast extract per liter of distilled water and had an unadjusted pH of 6.8. The acidity increased to pH 6.6 when 100 mg of unneutralized oleic acid was added per 50 ml of medium. Distilled water in the medium prevented the formation of insoluble soaps under alkaline conditions.

Routinely, growth and fermentations were done in 50 ml or 300 ml conical flasks containing 20 ml or 50 ml of YE medium, respectively. Flasks were closed with either cotton surgical sponges or cotton fiber coffee filters (Schwartz Mfg. Co., Two Rivers, Wis.) and the medium without added fatty acids was sterilized for 15 min at 121 C. Flasks were inoculated with one drop of a 24 hr broth culture and were incubated on a rotary shaker at 180 rpm at 28 C.

Although aerobic conditions were necessary for the growth of all organisms used, fermentations were done under both aerobic and anaerobic conditions. Maximum populations of 1 to 10 billion cells per milliliter developed within 16 hr in shaken flasks; the presence of fatty acids did not alter the extent of growth. Anaerobic fermentation conditions were achieved by replacing air with prepurified nitrogen gas (less than 8 ppm O_2) after growth was completed. Without oxygen, growth ceased and the fermentation was accomplished by resting cells. To achieve anaerobiosis, a sterile two hole rubber stopper carrying bead valves was substituted for the cotton stopper in the flasks; these valves were connected to a vacuum line and through a sterile cotton filter to the N_2 tank. After air was replaced by nitrogen, the flasks were returned to the shaker.

Either at the start or during the course of the fermentation, 100 mg of unsterilized fatty acid was added aseptically to each flask. In anaerobic fermentations, fatty acid additions were made in flowing N_2. Replicate flasks allowed removal of a flask at intervals as samples. Sampling by aliquots from a single flask was impractical because both substrate and product were partially insoluble and not uniformly dispersed.

Cells for 5-liter fermentations were grown in Fernbach flasks containing 715 ml of medium. The cells from seven flasks were aseptically transferred to a sterile 6 liter Florence flask carrying a teflon-coated bar magnet and a rubber stopper with bead valves and a sampling tube. Air was replaced with N_2, substrate was added and samples were taken under flowing N_2.

The pH of fermentation samples was taken and sufficient 6N H_2SO_4 then was added to lower the pH below 2 and immediately stop microbial activity. Samples containing polyunsaturated substrates were held at -20 C under N_2 until analysis. Extraction of, and later operations on such substrates were performed under N_2 to prevent autoxidation. Acidified samples were extracted continuously with ether for 6 hr. Teflon sleeves were inserted between standard taper glass joints to prevent interference in infrared analyses by silicone greases. Ether extracts were dried with anhydrous Na_2SO_4; the ether was evaporated and fatty acids were esterified with diazomethane.

The methyl esters of samples, controls and substrate standards were dissolved in hexane or acetone and injected into an F&M Model 700 gas liquid chromatograph (GLC) containing two 4 ft columns packed with 20% SE30 silicone on 80-100 mesh Chromosorb W, and equipped with a hydrogen flame ionization detector. Samples were injected at 250 C at a helium flow rate of 50 ml/min. After 1 min at 250 C, the temperature was programmed to rise 15°/min to 305 C. High temperatures initially used to facilitate survey work allowed satisfactory separations, except that 10-keto and 10-hydroxy fatty acids usually occurred as one asymmetric peak. Peak areas were determined with a recording Disc integrator. Hydroxy and keto acids and their mixtures were detected by internal reflection infrared analysis on a Beckman Model IR-8; the methyl ester was deposited as a film on the surface of a KRS-5 plate (Wilks Scientific Corp., South Norwalk, Conn.) by evaporation from a hexane solution. Thin layer chromatography (TLC) was done with a mixture of hexane-ether (9:1) on Eastman Chromagram silica gel sheets. Positions of fatty acid esters were determined by spraying with 0.2% 2',7'-dichlorofluorescein in methanol and marking under UV. Keto esters were identified by running duplicate samples on the same sheet and spraying one half of the divided sheet with 2,4-dinitrophenylhydrazine HCl, which gives yellow hydrazone spots without heating. Subsequent fuming with ammonia gave more easily detectable red brown keto spots and also revealed hydroxy compounds in daylight at least as well as by the fluorescein technique.

Pure crystalline hydroxy fatty acid esters were obtained by column chromatography on silica gel eluted with hexane-ether mixtures in steps from 3% to 50% ether. The pure hydroxy

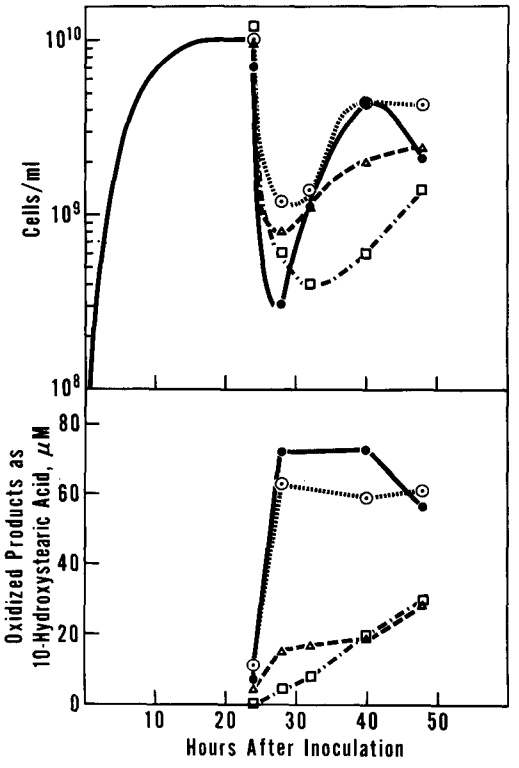

FIG. 1. Cell counts and product formation from aerobic fermentation of oleic acid by NRRL B-3266. A total of 350 μM oleic acid was added to each flask beginning with 70 μM at either 0 hr (•—•), 16 hr (0---0), 20 hr (Δ--Δ), or 24 hr (□-□); the balance was added at 24 hr.

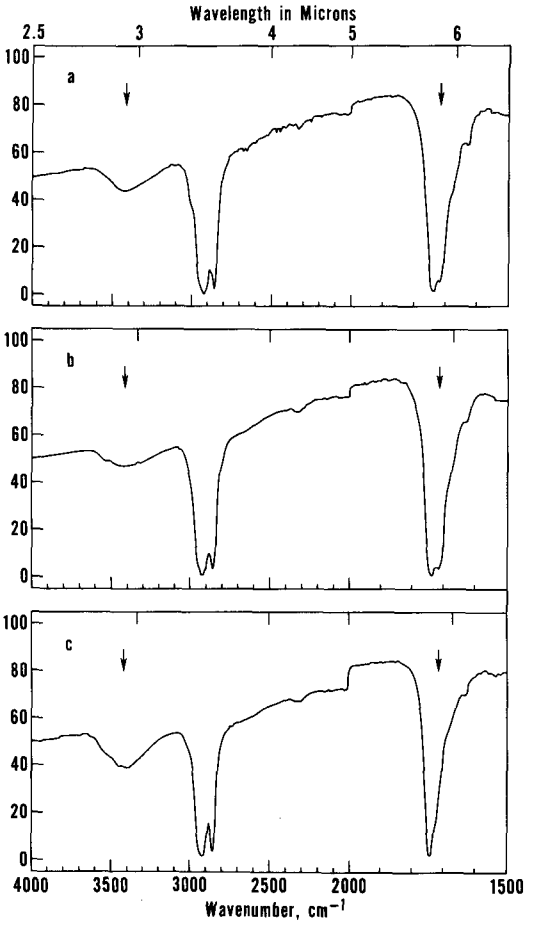

FIG. 2. Infrared analysis of 10-hydroxy- and 10-ketostearic acid formation from oleic acid by NRRL B-3266 (see Table I). Arrows show position of hydroxyl absorption at 3420 cm^{-1} and keto absorption at 1715 cm^{-1}. a. Aerobic, 28 hr sample, b. Aerobic, 40 hr sample, c. Anaerobic (under N_2 after 24 hr), 40 hr sample.

esters were used to determine optical activity at 26.5 C with a 1 dm path length in a Model 60 Cary recording spectropolarimeter. Mass spectra of the corresponding keto esters, prepared by chromic acid oxidation, were determined on a Model 12-90 Nuclide mass spectrometer. One mass spectrum (methyl 4-ketolaurate) was prepared from a GLC eluant passed directly into a Bendix Model 12 Time-O-Flight instrument. The spectra were interpreted according to the fragmentation scheme for keto esters of fatty acids (10).

RESULTS AND DISCUSSION

The capacity of about 130 bacterial soil isolates to modify fatty acid structure was investigated by growing the organisms aerobically in 20 ml of YE medium in the presence of a mixture of nonanoic, oleic and erucic acids. Gas chromatograms of methylated ether extracts were examined for nonsubstrate peaks. One organism produced a compound whose GLC peak corresponded to 10-hydroxystearic acid. Subsequently, a variant strain of this organism was obtained by repeated transfer in shaken medium; this strain was superior to the original isolate in forming hydroxystearic acid from oleic acid. Both strains were gram negative short rods that did not form pigment and were cytochrome-oxidase positive. They did not produce acid fermentatively from carbohydrate. The original isolate produced polar flagella, singly or in tufts of two or more, and was weakly motile. This strain has been entered in the ARS Culture Collection as NRRL B-3294. The variant strain was nonflagellated. Its properties remained stable either when lyophilized or when transferred on solid medium. This

TABLE I

Oxidized Products of Oleic Acid as Gas Liquid Chromatographic Peak Areas of Methyl Esters

Oleic acid added (mg)	Fermentor atmosphere	GLC[a] peak	NRRL B-2994 Sampled, hr 24	28	40	NRRL B-3294 Sampled, hr 24	28	40	NRRL B-3266 Sampled, hr 24	28	40
100 (0 hr)[b]	Air	a	1	--	1	3	--	1	5	--	1
0 (24 hr)		b	0	--	0	0	--	0	26	--	12
		c	6	--	4	0	--	0	1	--	5
0 (0 hr)	Air	a	--	74	62	--	58	50	--	66	39
100 (24 hr)		b	--	1	3	--	4	1	--	4	19
		c	--	3	5	--	1	1	--	1	1
20 (0 hr)	Air	a	--	62	54	--	59	40	--	21	21
80 (24 hr)		b	--	0	1	--	4	1	--	11	35
		c	--	1	2	--	1	1	--	1	3
20 (0 hr)	N_2after	a	--	58	37	--	46	10	--	56	21
80 (24 hr)	24 hr	b	--	14	19	--	44	74	--	38	47
		c	--	4	2	--	1	1	--	0	0

[a]a, Peak area of residual oleic acid; b, combined peak area of 10-hydroxy- and ketostearic acids; c, combined area of chain shortened keto acids.

[b]One hundred milligrams of oleic acid (as Me ester) has peak area normalized to 100 units.

variant was used for almost all the studies reported and is available as *Pseudomonas* sp. NRRL B-3266. Both strains are easily distinguishable from the polar flagellate NRRL B-2994 previously reported (2) which produces a water insoluble yellow pigment and is viscous on TGY slants. This organism also has been identified as a member of the genus *Pseudomonas*.

Oleic acid fermentations with NRRL B-3266 were conducted with variations in media, initial pH and temperature. Glucose was not beneficial, and the simple YE phosphate buffer medium was as effective as any tried. Although the organism grows well from pH 6 to 9, production of 10-hydroxystearic acid from oleic acid is not appreciably affected by initial pH. The unadjusted medium becomes slightly alkaline during growth in the presence or absence of fatty acids. Inclusion of Triton X-100 to enhance dispersion of fatty acids did not improve yields. No specific effect of temperature on the fermentation reaction was found between 20 to 38 C when equal numbers of cells were used, although the rate of cell division was accelerated at higher temperatures. The results reported were obtained at 28 C.

Under aerobic conditions, a mixture of 10-ketostearic acid and 10-hydroxystearic acid is formed by fermentation of oleic acid. Such a mixture was detected by spraying thin layer chromatograms with 2,4-dinitrophenylhydrazine to form hydrazones in situ, and by infrared analysis. In addition to 10-hydroxy- and 10-ketostearic acids, small amounts of other keto acids were found in ether extracts of fermentation liquors. These acids had lower GLC retention times, and a semilog plot of retention time vs. carbon number indicated chain-shortening by 2-carbon units; the methyl ester of one of these, 4-ketolauric acid, was identified by mass spectrometry. Such compounds would be expected to result from dehydrogenation and beta-oxidation of the initial 10-hydroxystearic acid product.

More product was formed at faster rates and less oleic acid was metabolically degraded if the cells were first grown in the presence of a small amount of oleic acid and if the principal quantity of substrate was added later. This suggests that formation of the responsible enzyme may be derepressed by the presence of substrate during cell growth. Results of further investigation of the role of inducer oleic acid are represented in Figure 1. At intervals during growth 70 μM of oleic acid was added to 50 ml of inoculated medium in different flasks to induce the enzyme; an additional 280 μM was added as fermentation substrate to all flasks at 24 hr. Individual flasks of each inducer series were removed at intervals; 1 ml samples were taken aseptically for plate counts and the rest was used for GLC assay. The culture achieved maximum populations in 16 to 20 hr. Apparently inducer fatty acid must be added before cell division ceases in order to obtain rapid production of hydrated product in high yield. The 90% reduction in viable cells upon the addition of fatty acid could result from a radical change in cell wall permeability caused by the fatty acid. Easier access of enzyme to substrate must be considered as an alternative to enzyme induction.

Undoubtedly the enzyme is intracellular;

TABLE II

Mass Spectral Data of Keto Products

Compounds	Parent mass	Fragments[a]	Mass
Me 10-ketostearate[b]	312	$^{1}\ \ \ ^{10}$ $CH_3OOC(CH_2)_8CO$	199
		$^{18}\ \ \ ^{10}$ $CH_3(CH_2)_7CO$	141
Me 10-ketopalmitate[b]	284	$^{1}\ \ \ ^{10}$ $CH_3OOC(CH_2)_8CO$	199
		$^{16}\ \ \ ^{10}$ $CH_3(CH_2)_5CO$	113
Me 4-ketolaurate[c]	228	$^{1}\ \ \ ^{4}$ $CH_3OOC(CH_2)_2CO$	115
		$^{12}\ \ \ ^{4}$ $CH_3(CH_2)_7CO$	141
Me 6-ketolaurate[c]	228	$^{1}\ \ \ ^{6}$ $CH_3OOC(CH_2)_4CO$	143
		$^{12}\ \ \ ^{6}$ $CH_3(CH_2)_5CO$	113

[a]Selected fragments that prove keto position. Numbers over fragments indicate carbon positions in parent compound.

[b]Obtained by chromic acid oxidation of the methylated 10-hydroxy acids formed by fermentation of oleic and palmitoleic acids with NRRL B-3266.

[c]Chain-shortened acids obtained as minor products from oleic and palmitoleic acid aerobic fermentations with NRRL B-3266.

centrifuged cells in buffer are active, whereas the supernatant is not. After sonication of washed cells, some oxidative activity on oleic acid is present in the supernatant obtained by centrifugation at 20,000 x *g*.

When induced resting cells are used, 10-hydroxystearic acid is formed anaerobically. Anaerobic conversion suggests that the hydroxyl oxygen is derived from water rather than molecular oxygen and that the enzyme is a hydratase (hydro-lyase) rather than an oxygenase. For the different bacteria studied, Table I gives the results of fermentations done with different gas atmospheres and variations in the time of oleic acid addition. The results are expressed as arbitrary GLC peak areas of methyl esters of residual oleic acid, the combined peak of 10-hydroxy- and ketostearic acids and the sum of chain-shortened keto acids. It is evident that an anaerobic reaction with induced resting cells gives the greatest yields with minimal loss of oleic acid. As mentioned earlier, strain NRRL B-3266 was selected for its ability to make more oxidized product aerobically than NRRL B-3294. Strain NRRL B-2994 forms little product aerobically under the conditions used; Wallen et al. (2) found that pH 8.8 and addition of Triton X-100 with the oleic acid at 24 hr afforded maximum yields during the much longer aerobic fermentation periods. Cell counts from anaerobic fermentations showed that populations remained stable for several hours after the addition of oleic acid at 24 hr; the population then decreased. Production of hydroxy acids also continued for extended periods.

Infrared bands for stretching frequencies of keto (1715 cm^{-1}) and associated hydroxy (3420 cm^{-1}) groups are shown in Figure 2. These spectra illustrate the apparent conversion of 10-hydroxy-to 10-ketostearic acid with time under aerobic conditions and the complete inhibition of such dehydrogenase activity anaerobically. TLC showed that several keto compounds were present in aerobic samples but not in anaerobic samples.

Undegraded fatty acid products from the fermentation of both oleic and palmitoleic acids (see below) were methylated and then oxidized with chromic acid to their keto deriva-

TABLE III

Melting Points of Hydrated Products

	10-Hydroxy-stearate	10-Hydroxy-stearate	10-Hydroxy-palmitate
Organism	NRRL B-2994	NRRL B-3266	NRRL B-3266
Acid	88.0-89.0 C[a]	87.5-88.0 C	72.0-72.5 C
Me ester	57.0-58.0 C[b]	57.0-57.5 C	44.0-45.0 C

[a]86-86.5 C (2), 86.5-87 C (8).
[b]53-54 C (2), 56.5-57 C (8).

tives. These keto esters then were subjected to mass spectrometry. Chain-shortened acids isolated from aerobic fermentations contained only keto groups; two of these were analyzed as their methyl esters. Table II shows the parent mass numbers obtained from the observed molecular ion peaks and those of peaks clearly corresponding to the expected fragments (10). These results confirm the placement of the oxygen on carbon 10 of the substrate acids and chain-shortening from the carboxyl end.

A number of unsaturated fatty acids and related compounds were tested as substrates for NRRL B-2994 and NRRL B-3266 by the induced resting cell technique under aerobic and anaerobic conditions. All fatty acids having *cis* unsaturation in the 9,10 position, regardless of chain length, gave a product of longer GLC retention time than the substrate. Alkenoic acids with *trans* configuration or no unsaturation in the 9,10 position were not altered. The following *trans* acids were tested: palmitelaidic (*trans*-9-hexadecenoic), elaidic (*trans*-9-octadecenoic), *trans*-vaccenic (*trans*-11-octadecenoic) and linelaidic (*trans*-9, *trans*-12-octadecadienoic). Inactive *cis* fatty acids tested were: petroselenic (*cis*-6-octadecenoic), *cis*-vaccenic (*cis*-11-octadecenoic), 5-eicosenoic (*cis*-5-eicosenoic), 11-eicosenoic (*cis*-11-eicosenoic), erucic (*cis*-13-docosenoic) and nervonic (*cis*-15-tetracosenoic).

In fermentation extracts methylated with diazomethane under the GLC conditions described, oleic acid (*cis*-9-octadecenoic) had a retention time of 5.3 min vs. 6.9 min for its fermentation product, 10-hydroxystearic acid; the palmitoleic acid (*cis*-9-hexadecenoic) peak appeared at 3.9 min vs. 5.3 min for 10-hydroxypalmitic acid and myristoleic acid (*cis*-9-tetradecenoic) was retained 2.8 min vs. 4.1 min for its product presumed to be 10-hydroxymyristic acid. A straight line plot of the log of the retention time of these three products against chain length suggests a homologous series.

Fermentation of linoleic (*cis*-9, *cis*-12-octadecadienoic), linolenic (*cis*-9, *cis*-12, *cis*-15-octadecatrienoic) and ricinoleic (12-hydroxy-*cis*-9-octadecenoic) acids under nitrogen with cultures grown in the presence of either substrate acids or oleic acid gave GLC peaks with about 40% longer retention times than their respective substrates, indicative of hydroxy acids. The 10-hydroxy monoenoic, dienoic and 10,12-dihydroxy acids, respecti-

TABLE IV

Corrected Specific Rotations of Methyl Esters of Hydrated Products

	10-Hydroxy-stearate	10-Hydroxy-stearate	10-Hydroxy-palmitate
Organism	NRRL B-2994	NRRL B-3266	NRRL-B-3266
nM	$[\alpha]$ c = 5.00	$[\alpha]$ c = 4.42	$[\alpha]$ c = 5.00
546	-0.12[a]	-0.09	-0.30
436	-0.30	-0.27	-0.60
408	-0.34	-0.32	-0.71
365	-0.42	-0.43	-0.94
313	-0.64	-0.61	-1.41

[a]$[\alpha]$ = -0.16, -0.32, -0.39, -0.51 and -0.83 (respective λ) (8).

vely, are presumed to be products of these three substrates. Autoclaved control cultures containing these polyunsaturated acids shaken either in air or under nitrogen did not give autoxidized products which could be confused with the presumed hydroxy acids. Further work with these and other substrates is in progress.

Methyl esters of *cis* 9-alkenoic acids were not hydrated. After fermentation, a small amount of free acids could be removed from the ether extract with 5% Na_2CO_3; presumably the organisms have some esterase activity. Similarly, there was evidence of weak lipase activity against triolein but no evidence of hydration of the intact fat; when the Na_2CO_3-extracted triolein was saponified and esterified, only methyl oleate was found by GLC. Oleyl alcohol was inactive as a substrate with either oleic acid or itself as inducer. These results indicate that a free carboxyl is required for product formation.

A 5 liter fermentation with oleic acid was run by growing the cells in seven Fernbach flasks on a Gump shaker. Each flask contained 715 ml of YE medium and 0.165 g of acid. After 20 hr, the grown cultures were transferred aseptically to a sterile 6 liter stoppered Florence flask containing a magnetic stirrer. Air was replaced with nitrogen and 13 g of oleic acid was added. The culture liquor was stirred at a speed to give a shallow vortex. Insoluble flakes of product were apparent within a few minutes. Stirring was continued at 28 C for 36 hr. Concentrated H_2SO_4 was added slowly to pH 1.8, Hyflo Super-Cel was added and the fermentation liquor was vacuum-filtered on paper coated with filter aid. The filtrate was free of product and all but a trace of substrate acids. The filter cake was extracted twice with hexane, and the product was crystallized from the extract at -15 C. From oleic acid, crude crystalline product was obtained in 71 mole % yield, calculated as 10-hydroxystearic acid. A comparable fermentation was done using 1.15 g of palmitoleic acid during the aerobic growth phase and 10 g of acid for the anaerobic fermentation phase. Crude crystalline product was obtained in 53 mole % yield, calculated as 10-hydroxypalmitic acid.

The methyl esters of these acids were processed in 1 g batches on a 4 X 80 cm silica gel column. First, 1150 ml of 3% ether in hexane was passed through the column followed by a 10% ether mixture until tests on 25-ml fractions indicated that all 10-keto esters were removed and that 10-hydroxy esters had started to elute. The 10-hydroxy esters then were eluted with a 50% ether-hexane mixture.

The purified 10-hydroxystearic and 10-hydroxypalmitic acids and their methyl esters were used for determination of melting points and optical activity. Tables III and IV list these data in comparison with those for 10-hydroxystearic acid produced by NRRL B-2994. This comparison indicates that the 10-hydroxystearic acid formed by NRRL B-3266 is identical with that for NRRL B-2994. The melting points and optical activity of 10-hydroxypalmitic acid and its methyl ester have not been previously reported. The negative rotation of 10-hydroxystearate correlates with a D configuration of the hydroxyl substituent and 10-L-hydroxystearic acid has an equivalent positive rotation (8). By analogy and considering that the 10-hydroxypalmitate and stearate are here produced by the same highly specific enzymatic reaction, it is assumed that the (-)-10-hydroxypalmitate has the D configuration.

ACKNOWLEDGMENT

Spectropolarimeter data by J. E. Cluskey; mass spectrum of methyl 4-ketolaurate by E. Selke.

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[Received December 20, 1968]

Neutral Lipid and Fatty Acid Composition of Earthworms (*Lumbricus terrestris*)

J. CERBULIS [1,2] and M. WIGHT TAYLOR, Department of Biochemistry & Microbiology
Rutgers, The State University, New Brunswick, New Jersey 08903

ABSTRACT

Earthworms (*Lumbricus terrestris*) were extracted with chloroform-methanol (2:1) and examined primarily for neutral lipids and fatty acids. TLC showed spots for sterols, hydrocarbons, free fatty acids, phospholipids and pigments but none for glycerides (tri-, di- or mono). Saponification of the crude lipid extract yielded 32% fatty acids, 25% unsaponifiables and 43% unidentified. The lipid contained 3% hydrocarbon and 16% sterols. GLC of the hydrocarbons showed at least 13 components. GLC of the sterol fraction showed peaks corresponding to cholesterol (the major component), γ-sitosterol, β-sitosterol, stigmasterol, campesterol, and ergosterol. GLC showed that at least 38 fatty acids were present, with 18:1, 18:2, 18:0, 20:1 and 17:0 predominanting.

INTRODUCTION

Earthworms have been used for centuries in many countries as an antipyretic (1,2) and as a food. A recent report by Kobatake (3) showed that earthworm extracts contain antituberculous activity.

Some older reports are available about the composition of lipids of earthworms but this information is limited. It has been reported that fresh earthworms contain 1.2-1.3% of ether-soluble matter (4) and that this material is very dark in color (4,5) and has a sharp odor (5). The fat fraction contains 21.3-51.4% of unsaponifiable matter (1,4-6), a considerable amount of sterols and some nonesterifiable matter (4). Common triglycerides are not an important constituent of earthworm lipids (4).

This paper describes the initial work on the analyses of the earthworm fat fraction using modern methods.

EXPERIMENTAL PROCEDURES

Earthworms

The earthworms, *Lumbricus terrestris*, were collected at Boyertown, Pennsylvania, by digging them from the ground or by picking them up at night on the ground after rain. In each experiment 0.5-3 kg of live earthworms were washed with distilled water, then kept alive in beakers for 4 hr to eliminate soil particles from the digestive tract, then washed again with distilled water.

Lipid Extraction

The earthworms were extracted for 24 hr with chloroform-methanol (2:1 v/v) with occasional stirring using 20 ml of solvent per gram of fresh earthworms as described by Folch et al. (7). All solvents used were reagent grade, redistilled before use through Vigreaux-type column. The samples were extracted four times. Portions of the extracts were evaporated to dryness either on a steam bath with a stream of nitrogen or under reduced pressure at 35 C. The residue was redissolved in chloroform-methanol (2:1 v/v). and water-soluble impurities were removed by the method of Folch et al. (7). The chloroform layer was washed once with water. The washing was added to the aqueous phase and the latter was extracted once with chloroform. The chloroform layer and the aqueous layer were evaporated to dryness and the residues weighed.

Thin Layer Chromatography

This procedure was carried out as described by Cerbulis and Ard (8). Silica Gel G plates (Brinkman Instruments, Inc., Westbury, N.Y.) were reactivated at 105 C for 1 hr. Samples were applied at 2 cm intervals and developed in one-dimensional chromatography. The developing solvents were petroleum ether-diethyl ether-acetic acid (90:10:1 v/v/v) and benzene for neutral lipids and chloroform-methanol-water (65:25:4 v/v/v) for phospholipids. Iodine vapor was used to make the lipids visible.

Column Fractionation

Chromatographic columns with replaceable coarse-porosity sintered glass disk and Teflon stopcock were used. The lipid fraction was separated on a silicic acid column as described by Rouser et al. (9). The washed total lipid extract was applied to the column dissolved in chloroform. The 0.52 g of lipid fraction was applied to 2.5 x 30 cm column. Eluting solvents were:

[1]Eastern Regional Research Laboratories, USDA, Philadelphia, Pa. 19118.

[2]Abstracted in part from the Ph.D. dissertation of J. Cerbulis, Rutgers, The State University, 1966.

TABLE I

Chromatography of Earthworm Lipid Fraction on a Silicic Acid Column

Eluting solvent	Fraction weight, g	Original material, %	Components
Chlorofrom	0.2514	51	Pigments, sterols, free fatty acids
Chloroform-acetone (1:1)	0.0357	7	Pigments, glycolipids
Acetone	0.0046	1	Pigments, glycolipids
Methanol	0.1314	25	Pigments, phospholipids
Methanol-acetic (2:1)	0.0816	16	Pigments, phospholipids

chloroform, chloroform-acetone (1:1), acetone, methanol and methanol-acetic acid (2:1). The total recovery was 98.3%. Components were identified by TLC using known specimens. No attempt was made to determine the precise amount of the several components in each fraction. For results see Table I.

Saponification

The unwashed total lipid extract was saponified to avoid possible loss of fatty acids and probably some of the unsaponifiables. A 20 g sample was saponified with 1 N KOH in methanol under reflux for 75 min then an equal volume of water was added and the mixture extracted with petroleum ether to give the unsaponifiable fraction. The pH was then lowered to 1.0 with HCl and the fatty acid fraction extracted with petroleum ether leaving a dark brown-black aqueous material. The aqueous phase was extracted with diethyl ether to obtain the diethyl ether extract (see Unknown acid) leaving a diethyl ether-insoluble black aqueous material.

Fractionation of Unsaponifiable Material

This material was a slightly yellowish solid. Thin layer chromatography (TLC) showed spots corresponding to hydrocarbons, pigments and sterols, with sterols as the major group. To separate these materials in sufficient quantities for further analyses the fraction was chromatographed on a silicic acid column (Mallinckrodt, chromatographic grade) activated for 12 hr at 120 C and packed in a column, 4.2 cm i.d. to a bed height of 38 cm. The lipid was eluted as shown in Table II. The hydrocarbon-pigment fraction (I in Table II) was rechromatographed on a silicic acid-Hyflo Supercel (3:1, by weight) column. The mixture of adsorbents was slurried into the column with petroleum ether. The sample, dissolved in petroleum ether, was then added. Hydrocarbons were eluted from the column with petroleum ether almost at the solvent front as a colorless oil, which solidified at 0 C as a colorless mass. The oil was analyzed by gas chromatography. Diethyl ether and methanol removed all pigments from the column.

TABLE II

Chromatography of Unsaponifiable Earthworm Lipids on Silicic Acid Column

Fractions in order of elution	Eluting solvent	Vol. of eluting solvent, liter	Fraction weight, g	Original material, %	Ingredients
I	Petroleum ether	1.8	3.2	18	Hydrocarbons and pigments
	1% diethyl ether in petroleum ether	2.2			
II	2% diethyl ether in petroleum ether	6.7	12.8	73	Sterols and pigments
	4% diethyl ether in petroleum ether	10.0			
	10% diethyl ether in petroleum ether	8.5			
III	50% diethyl ether in petroleum ether	5.0	2.0	9	Pigments
	diethyl ether	4.0			
	Methanol, absolute	3.0			

The sterol-pigment fraction, which came off with increasing concentrations of diethyl ether (II in Table II), was evaporated to dryness and redissolved in a small volume of petroleum ether (bp 40-60 C) and left overnight at -30 C (10). The white crystals of precipitated sterols were removed by filtration and analyzed by gas liquid chromatography. TLC showed that the sterol precipitation was complete and that the petroleum ether contained pigments only.

Methylation of Fatty Acids Soluble in Petroleum Ether

Fatty acids were esterified with BF_3-MeOH complex (11). The fatty acid fraction (1 g) was added to the reagent (50 ml) and heated at 100 C for 3 min; then the mixture was poured into water and extracted with diethyl ether. The diethyl ether extract was washed once with 5% sodium bicarbonate, twice with water, dried over $MgSO_4$ and the solvent removed. TLC showed that esterification was complete.

During methylation pigment color of the fatty acid fraction changed from yellow to dark brown to black. The methyl ester fraction was purified on a 1.8 x 25 cm column of Silica Gel G (which had been heated at 118 C for 3 hr), using benzene as a solvent. Methyl esters were eluted as a yellowish fraction close to the solvent front, then the pigment fraction followed slowly. TLC, using Silica Gel G plate with benzene as solvent, showed that the separation was complete.

Gas Chromatography of Fatty Acid Methyl Esters

The gas chromatogram was run using 8% (w/w) EGSS-X organosilicone polyesters (Applied Science Laboratories, State College, Pa.) on a 100-120 Gas-Chrom P column. Temperature programming was not used because of excessive baseline rise. Instead, three isothermal runs were made at 190 C, 150 C and 125 C respectively. Quantitative results for several standard methyl ester mixtures (Applied Science Laboratories) were determined. Peak areas were calculated by the peak height multiplied by peak width at half peak height method (12) but were expressed as weight per cent (area per cent).

RESULTS

Lipid Content of Earthworm

Lipid content (based on fresh weight) varied according to the season when the earthworms were collected. The lowest content (0.65%) were found in March and April, and the highest (3.5%) in May. The average content of lipids was 1.0-1.5%.

Separation of the chloroform-methanol (2:1) extract with added water as described by Folch et al. (7) resulted in a chloroform layer (lipids), 45%, and a methanol-water layer (nonlipids), 55%, of the total extract weight (see Lipid Extraction). However, a spot test using Rhodamine 6G reagent showed that the aqueous layer gave a Rhodamine 6G positive reaction, showing the presence of some lipids. TLC and paper chromatography showed the absence of those lipids found in the chloroform layer but the presence of pigments and ninhydrin positive material, most of which were fluorescent under ultraviolet light.

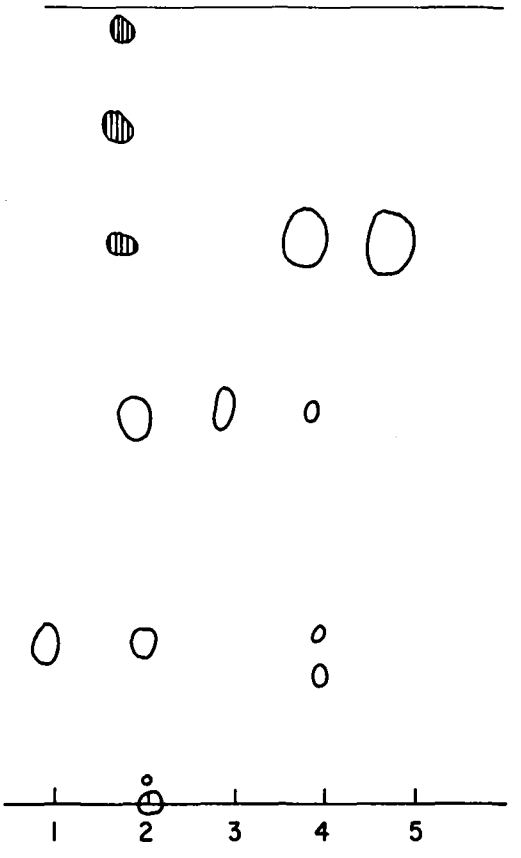

FIG. 1. Thin layer chromatogram of earthworm neutral lipids 400 μg lipids/spot. Solvent: Petroleum ether-diethyl ether-acetic acid (90:10:1) 1, Cholesterol; 2, earthworm neutral lipids; 3, palmitic, oleic, stearic acids; 4, hazel nut oil,; 5, triglycerides. Reagent: iodine vapor.

The lipid fraction was separated on the silicic acid column, as summarized in Table I. Pigments were present throughout all fractions.

Properties and Composition of Lipid Fraction

The lipid fraction was brown-black in color and had a sharp odor. TLC showed no triglycer-

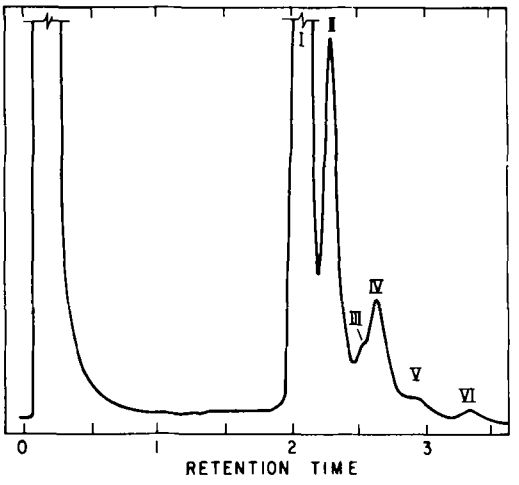

FIG. 2. Gas liquid chromatogram of earthworm sterols I Cholesterol, II Ergosterol, III Campesterol, IV γ-Sitosterol, V Stigmasterol, VI β-Sitosterol, Barber-Colman Model 10 instrument with the argon ionization detector with 6 ft. x ¼ in. i.d. column of Gas Chrom-P (coated with dimethyldichlorosilane) 80-100 mesh, with the flow rate 60 ml N_2/min at 212 C columns, 275 C cell and 305 C flash heater.

ides, diglycerides or monoglycerides. Free fatty acids, phospholipids, glycolipids, sterols and pigments were the compounds present (Fig. 1).

Saponification of 20 g of the total lipid extract gave the following results: unsaponifiable material 25%, petroleum ether-soluble fatty acids 32%, and a petroleum ether-insoluble residue, very dark in color, 43%. These data vary somewhat from the value of 55% water-soluble material for the total lipid extract which was not saponified. Apparently saponification liberated material to the petroleum ether-soluble fractions.

The unsaponifiable fraction was found to have the following composition: hydrocarbons 15%, pigments 15-20%, sterols 65-70%.

Hydrocarbons

A sample of hydrocarbon material was gas chromatographed on a 4 foot column of Apiezon L on 60/80 mesh glass beads with the flow rate 60 ml N_2/min. Identification was only tentative because standard specimens were not available. At least 13 hydrocarbon peaks were observed, including straight chain and branched chain compounds (13).

Sterols

The sterol fraction m. 138 C, was analyzed by gas liquid chromatography (14) on a 6 foot column of Gas Chrom P, 80-100 mesh (Applied Science Laboratories, State College, Pa.) with the flow rate 60 ml N/min. At least 6 sterols were present (Fig. 2). Cholesterol, γ-sitosterol, β-sitosterol, stigmasterol and campesterol were identified by comparison with known specimens. Cholesterol was the major component. The second largest component, probably ergosterol, was not unequivocally identified due to lack of a known specimen.

The Composition of the Fatty Acid Fraction

The fatty acid fraction was very complicated as shown in Table III. At least 38 peaks were present in the chromatogram and several more fatty acids were undoubtedly present because some peaks overlapped each other and represented two or more acids. Acids from C_{11} to C_{27} were present, with the C_{18} and C_{20} fatty acids predominating. Some of the anteiso branched chain saturates could be iso unsaturated compounds. Hydroxy, epoxy and cyclo fatty acids also could be present. The present data confirm and extend those of Lovern (4), who reported the presence of C_{10} to C_{22} even fatty acids.

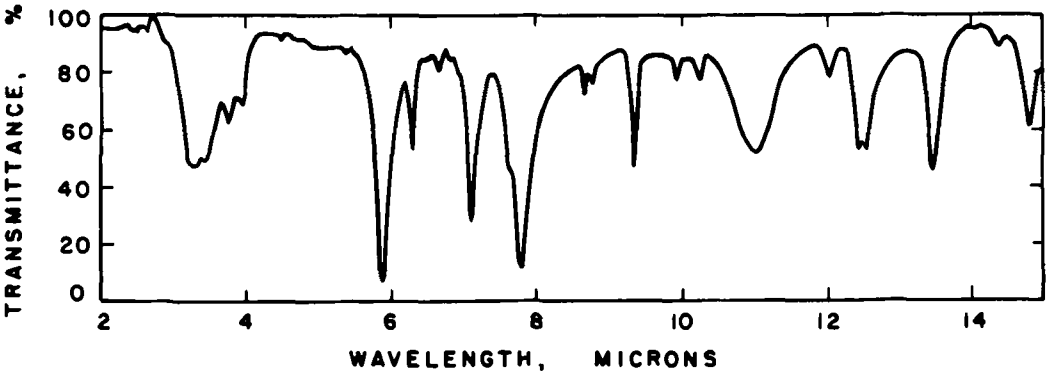

FIG. 3. Infrared spectra of unknown acid. Prepared as KBr pellets, taken on a Beckman IR-4 infrared spectrometer.

TABLE III

Fatty Acid Composition, Weight Per Cent of Total Acids

Fatty acid	Carbon number	Weight, %
10:0	10.0	–
11:0	11.0	0.1
11:1	11.5	0.2
12:0	12.0	1.3
12:1	12.5	0.9
13:0	13.0	0.5
13:0	12.3	0.2
13:0	12.7	0.5
13:1	13.5	0.5
14:0	14.0	1.2
14:0	13.3	0.2
14:1	14.5	2.8
14:2	15.1	1.6
15:0	15.0	
15:0	14.3	0.6
15:0	14.7	0.9
15:1	15.5	1.5
16:0	16.0	2.8
16:0	15.3	0.3
16:0	15.6	0.8
16:1	16.5	2.0
16:2	17.3	1.6
18:0	17.5	
16:4 (6,9,12,15)	18.9	2.6
17:0	17.0	6.5
18:0	17.1	
17:0	16.3	0.6
17:0	16.6	1.2
18:0	18.0	7.7
18:1	18.4	12.4
18:2	19.2	5.1
19:0	19.0	–
19:0	18.2	1.0
19:0	18.6	1.2
20:0	20.0	0.3
20:0	19.5	0.1
20:1	20.4	8.7
20:2 (8,11)	21.1	1.0
20:2 (11,14)	21.3	2.7
21:0	21.0	1.1
22:0	22.0	3.2
22:0	21.8	
22:1	23.4	4.2
23:0	23.0	1.9
23:0	23.9	
24:0	24.0	1.8
24:0	23.6	4.9
24:1	24.5	0.5
25:0	24.9	0.6
26:0	25.4	0.5
27:0	27.0	9.7

Phospholipids

TLC showed that the phospholipid fraction contained several components.

Unknown Acid From the Petroleum Ether-Insoluble Material

The diethyl ether extract from the petroleum ether-insoluble material from the fatty acid fraction was concentrated to a small volume and an equal volume of petroleum ether added to the solution. This solution was left at 0 C for a week, during which time a crystalline solid (0.12 g; from 20 g of the total lipid extract) gradually precipitated. This solid was a strong acid with no sharp melting point, but turned slowly brown at 195 C and decomposed at 235 C. Elemental analysis (performed by Carl Tiedcke, Teaneck, N.J.) gave C, 57.54%; H, 3.32%; N, 0.00%. The molecular weight was 126, which gave the empirical formula $C_6H_{4\text{-}6}O_3$. $FeCl_3$ gave a reddish-brown color. There was strong absorption in the ultraviolet region: peaks were at 200, 274, 280 mμ in MeOH; 228, 270, 290 mμ in NaOH, and 205, 228, 278 mμ in H_2SO_4. The infrared absorption spectrogram (Fig. 3) gave the characteristic COOH bands at 3.2-3.4 μ, 5.8-5.9 μ, 11.0 μ and 14.8 μ, and this COOH was not conjugated. There was a band for -C=C-C=C- at 6.3 μ and for C-O-C at 7.5 μ, indicating that such components were present in a ring system, probably of the furan type. The compound was not identified, but was probably a mixture of an acid of the enol-ketol type and its ester. Paper chromatography showed only one spot.

Pigment Fraction

The pigment fraction exhibited steeply rising absorption in the ultraviolet. Further fractionation of pigments gave a small fraction with an absorption similar to carotenoid pigments but none of the peaks was characteristic to known carotenoids. One of these pigments gave an absorption curve identical to that of vitamin K_5 in the ultraviolet region. The absorption curves of earthworm pigment fractions were similar to those obtained for the marine *Annelida* worm (*Thoracophelia mucronata*) (15).

When chloroform soluble lipid fraction was treated by milk alkaline methanolysis based on the method of Ballou et al. (16), the pigments again remained in the chloroform phase, leaving the upper aqueous phase almost colorless.

DISCUSSION

The absence of glycerides in fat fraction and the brown-black color of fat fraction are two of the most characteristic properties of earthworm fat. Little information could be found in the literature about them. The black fat phenomenon possibly is characteristic of *Annelida* since another species, *Arenicola marina*, has also been reported to contain black fat (17). Brown fat is characteristic of mammalian hibernators (18) and the earthworm is an invertebrate hibernator.

The earthworm inhabits soil rich in peat or organic matter and swallows and excretes large quantities of soil. This organic matter, or humus is separated in two groups of com-

pounds: humic substances, a series of brown to black polymers, and nonhumic substances, consisting of compounds such as amino acids, carbohydrates and lipids (19). An open question remains: is black fat characteristic of creatures inhabiting soil rich with organic matter?

Lipid content of humus is 1.2-6.3%. These lipids are originated from undecomposed plant residues and the bodies of living and dead microfaunal organisms. They are very stable in soil and contribute a large part of petroleum (20).

It seems very likely that the soil humus is the primary source of earthworm pigments, sterols, hydrocarbons and free fatty acids, because these compounds are part of earthworm diet.

Gregarines (*Protozoa, Sporozoa*) are common parasites in the earthworm digestion system (21) and probably also affect the chemical composition of warthworms (22).

The almost complete absence of glycerides in earthworm fat fraction is unusual. It is not an artifact of isolation because several solvent systems were used for the extraction of lipids with identical results: no triglycerides were present. When the same solvent systems were used for the extraction of lipids from dried plant material, triglycerides were always present.

Absence of triglycerides could suggest that earthworms have other unidentified types of storage lipids.

ACKNOWLEDGMENT

We thank the U.S. Public Health Service for a Training Grant 2-G-642 and the American Cancer Society for a Grant-in-Aid. W F. Carey of the Office of the State Chemist of New Jersey and J Eisner, Food and Drug Administration performed gas liquid chromatography of hydrocarbons and sterols. A. J. Menna of the U.S. Department of Agriculture prepared the Figures and N. Nicolaides of the University of Southern California made review and comments.

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[Received May 1, 1968]

Steroids in Bovine Muscle and Adipose Tissue

C. TU[1], W. D. POWRIE and O. FENNEMA, Department of Food Science
University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT

Thin layer and gas liquid chromatography, (GLC) were employed as complementary techniques to investigate naturally-occurring steroids in the unsaponifiable matter of bovine muscle and adipose tissue. Three GLC liquid phases, differing in selective partition properties, were used to effectively identify unknown steroids. The results indicate that cholesterol and minor amounts of desmosterol, Δ^7-cholestenol, lanosterol, dihydrolanosterol, dehydromethostenol, Δ^8-methostenol, Δ^7-methostenol, cholestanol and possibly ergosterol were present in the bovine tissues. The minor steroids, with the exception of cholestanol and ergosterol, are steroid precursors in cholesterol biosynthesis. Common hormonal steroids were not found in the unsaponifiables of the tissues.

INTRODUCTION

The unsaponifiable matter of skeletal muscle and adipose tissue from domesticated animals consists of steroids, hydrocarbons and fat-soluble vitamins (1,2). Tu et al. (3) estimated the average total cholesterol content of beef and pork muscles to be 58 and 65 mg/100 g, respectively. Extramuscular adipose tissue from beef and pork carcasses contained between 47 and 68 mg/100 g of total cholesterol. Little attention has been directed towards the determination of other steroids as naturally occurring compounds in muscle and adipose tissue. Recently, Williams and Pearson (2) reported that lanosterol, 7-ketocholesterol and cholesterol were in the unsaponifiable fraction of pork fat. According to Tu et al. (3), 7-hydroxycholesterols and 7-ketocholesterol were not present in the lipid extracts from cooked chuck patties. Since several studies have indicated that skeletal muscles from various mammals contain enzymes which can metabolize hormonal steroids (4-7), it was presumed that hormonal steroids are present as naturally occurring constituents of muscle. Recently Dietschy and Siperstein (8) demonstrated the biosynthesis of cholesterol in rat skeletal muscle incubated in an acetate solution, but identification of the steroid precursors of cholesterol was not carried out. Moreover, Durr (9) pointed out that cholesterol could be biosynthesized in adipose tissue. Thus a number of metabolic steroids would be expected in the muscle and adipose tissues of the steer.

The objective of this study was to identify steroids in the unsaponifiable matter of muscle and adipose tissue of the steer.

EXPERIMENTAL PROCEDURES

Materials and Methods

Bovine muscle from the round of steer carcasses and adjacent superficial adipose tissue were selected. Prior to moisture analyses and lipid extraction, muscle and adipose tissue samples were cut into small pieces and ground twice in a Hobart grinder with a plate having 0.5 cm holes.

Steroids used as reference standards were from a variety of sources. Δ^8-Methostenol and zymostenol were provided by A. A. Kandutsch and G. J. Schroepfer, Jr. Δ^7-Methostenol was obtained from W. W. Wells. 14α-Methyl-5α-cholest-7-en-3β-ol was supplied by J. C. Knight. Cholesterol, cholestane, androstane, 5α-cholestane-3β-ol, desmosterol, β-sitosterol, stigmasterol, cholestan-3-one, cholesteryl palmite, progesterone, estradiol, estrone, androstan-17-one, testosterone and androsterone were obtained from Applied Science Laboratories, State College, Pa. Lanosterol, dihydrolanosterol, Δ^4-cholesten-3-one, $\Delta^{3,5}$-cholestadiene-7-one, ergosterol, Δ^5-androsten-17α-methyl-3β, 17β-diol, cholestane-3β, 5α, 6β-triol, 5α-pregnan-3, 20-dione and 5α-pregnan-21-ol-3,20-dione were purchased from Steraloids Inc., Pawling, N. Y. Δ^7-Cholestene-3β-ol was obtained from Ikapharm, Israel. Squalene was purchased from Eastman Kodak, Rochester, N. Y. N-octacosane and Δ^4-androsen-3,17-dione were obtained from Analabs, Hamden, Conn. The α-tocopherol was purchased from Signa, St. Louis, Missouri.

Lipid Extraction

Lipid was extracted from 100 to 300 g of mascerated muscle or adipose tissue by a method outlined by Tu et al. (3).

Saponification

Solvent in each lipid extract was evaporated under reduced pressure in a flask rotated in a water bath at 50 C. Each lipid residue was

[1]Postdoctoral research associate.

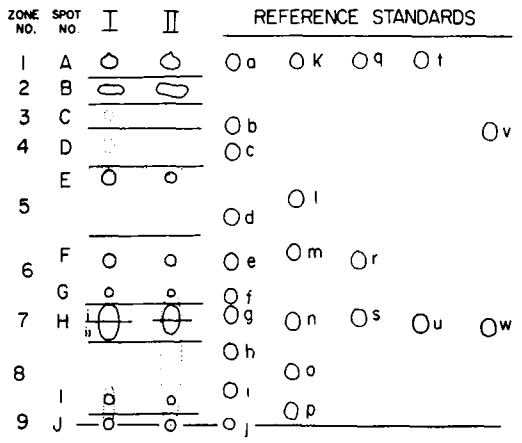

FIG. 1. Rhodamine 6 G-silica gel TLC of unsaponifiable matter for muscle (I) and adipose tissue (II). Solvent system: benzene-ethyl acetate (10:1 v/v). Reference standards: a, squalene; b, cholesteryl acetate; c, $\Delta^{3,5}$-cholestadiene-7-one; d, cholestane-3-one; e, lanosterol; f, Δ^{8}-methostenol and Δ^{7}-methostenol; g, cholesterol; h, estrone; i, estradiol; j, cholestane-$3\beta,5\alpha,6\beta$,-triol; k, cholesteryl palmite; l, androstane-17-one; m, Δ^{4}-cholestene-3-one; n, ergosterol; o, progesterone; p, testosterone; q, n-octacosane; r, dihydrolanosterol; s, desmosterol; t, cholestane; u, cholestanol; v, α-tocopherol; w, Δ^{7}-cholestenol.

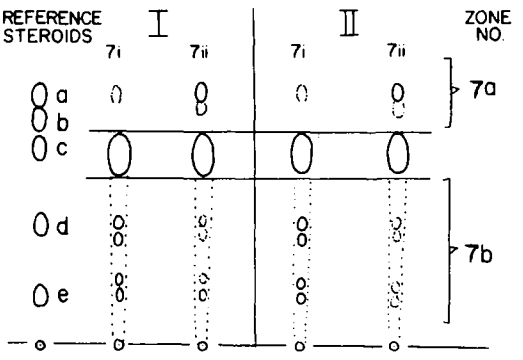

FIG. 2. $AgNO_3$ TLC of zone 7 (Fig. 1) eluates for muscle (I) and adipose tissue (II): 7i = eluate from upper half of zone 7; 7ii = eluate from lower half of zone 7. Solvent system: chloroform-acetone (95:5 v/v). Reference standards: a, cholestanol, b,Δ^{7}-cholestenol; c,cholesterol; d, desmosterol; e, ergosterol.

mixed with 10% KOH in ethanol (about 19 ml of KOH solution per gram lipid) and the mixture was held under nitrogen at 25 C for 14 hr with occasional stirring. Saponification by this method shall be referred to as cold saponification. Hot saponification was carried out by refluxing the KOH lipid mixture for 1.5 hr.

Water, twice the volume of added KOH solution, was added to the mixture and the resulting solution was extracted three times with 0.7 volume of ethyl ether. The combined ether extracts were washed three times with 0.5N KOH to remove any remaining free fatty acids and several times with water to neutrality. The washed extract was dried over anhydrous sodium sulfate. The solvent in the extract was evaporated under reduced pressure. An appropriate volume of chloroform was added to the unsaponifiable matter for thin layer chromatography (TLC) (1 ml of chloroform per 0.12 g of unsaponifiable matter).

Thin Layer Chromatography

Preparative TLC was carried out on 20x20 cm glass plates, each coated with a 0.5 mm layer of silica gel (Adsorbosil-2, Applied Science Laboratories, Inc.). A slurry was prepared by mixing 1 part of silica gel powder and 1.5 parts of water. Usually Rhodamine 6 G (Allied Chemical Corp., N.Y.) was added to the water to make a 0.1% dye solution prior to slurry preparation. The inclusion of Rhodamine 6 G was helpful for visualization of spots across developed plates. The slurry was applied to the plates by a Desaga-Brinkmann adjustable applicator. Each plate was activated at 110 C for 1 hr. The preparative TLC plates were spotted at 0.5 cm intervals along the origin with a chloroform solution of unsaponifiable matter by a Hamilton microsyringe. Each plate contained about 17 mg of the unsaponifiable fraction. A solvent system of benzene-ethyl acetate (10:1 v/v) was used to develop the plates. The plates were dried at 25 C. Spots on the Rhodamine 6 G plates were visualized with the aid of UV light as pink-yellow flourescent areas or as dark areas. The positions of the spots on dye-free TLC plates were determined by spraying a narrow region at the ends with 50% H_2SO_4 and then visualizing with UV light.

Each developed plate was divided into 9 zones, each containing one or more spots. The silica gel in each zone was scraped into a sintered-glass funnel for elution of the unsaponifiable components. The silica gel with Rhodamine 6 G from each of the upper seven zones was extracted three times with 15 ml of chloroform. The Rhodamine 6 G remained on the silica gel. With dye-free TLC plates, the components from zones 8 and 9 were eluted from silica gel with chloroform-methanol (1:1). For GLC studies, the solvent was evaporated under reduced pressure and the residue was dissolved in either methylene chloride for residues from zones 1 to 7 or methylene chloride-methanol (9:1) for residues from zones 8 and 9.

With the possibility that the broad-spreading

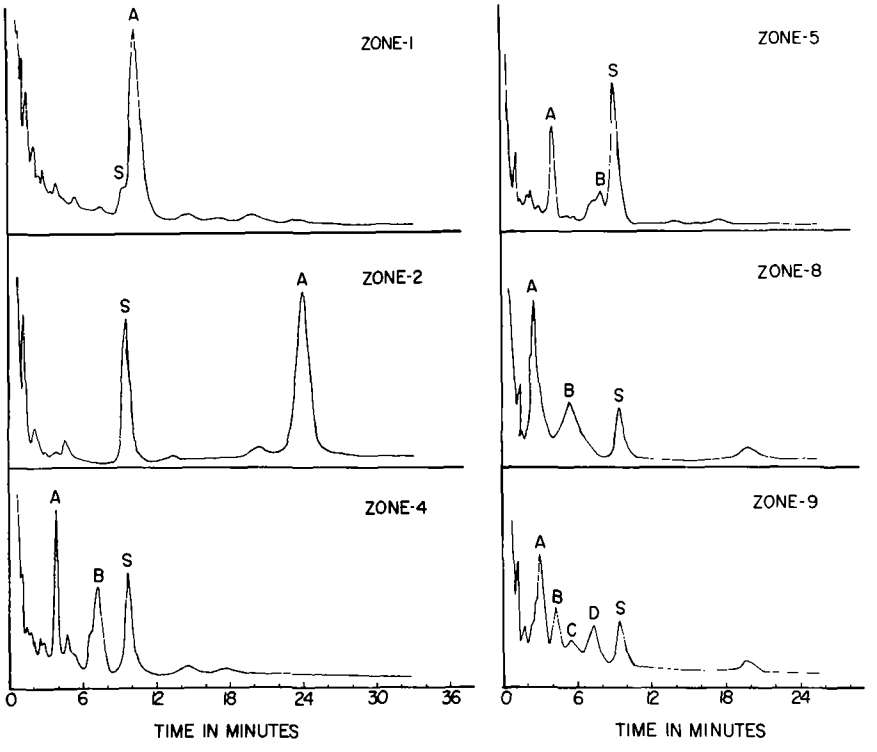

FIG. 3. Gas chromatograms of muscle unsaponifiables in zone eluates from TLC plate (Fig. 1). Operating conditions: 6 ft x 4 mm i.d. column with 1% SE-30 on Gas-Chrom Q, 80/100 mesh; column temperature, 211 C; 95 ml/min N_2 carrier gas; hydrogen flame detector. S represents the peak of added cholestane as reference standard.

zone 7 consisted of minor steroids as well as cholesterol, silver nitrate-silica gel TLC plates were used to separate them from the predominant cholesterol when the zone 7 eluate was applied. A slurry was prepared by mixing 60 g of silica gel powder (Adsorbosil-2) with 90 ml of 12.5% silver nitrate solution. This amount of slurry was sufficient to coat five 20x20 cm chromatoplates, each 0.5 mm thick. The chromatoplates were allowed to dry at 25 C for more than 5 hr in the dark. Each plate was activated at 110 C for 1 hr. For preparative TLC, the plates were spotted along the origin at 0.5 cm intervals. A solvent system of chloroform-acetone (95:5 v/v) was used to develop the plates. The plates were dried at 25 C. For qualitative evaluation, the plates were sprayed with 50% H_2SO_4 for visualization of spots. Spots on preparative plates were detected nondestructively by lightly spraying each dried plate with distilled water. When the plate was viewed against a dark background, the spots appeared white as the plate dried. Each plate was divided into two zones, one zone (7a) above the cholesterol band and the other (7b) between the origin and the cholesterol band. A method similar to that of Truswell and Mitchell (10) was used for steroid extraction. Adsorbent in each zone was scraped into a 15 ml conical centrifuge tube and extracted with 3 portions of 6 ml 50% ethanolic ammonia. The tubes were centrifuged at about 1000 x g after each extraction and the extractant was removed. The combined extracts were diluted with an equal volume of water and extracted three times with 15 ml redistilled n-hexane. The combined hexane extracts were washed four times with 25 ml of water and dried over anhydrous sodium sulfate. The solvent in the extract was evaporated under reduced pressure and the residue was dissolved in methylene chloride for gas liquid chromatographic (GLC) analysis.

R_c is defined as the distance of the sample spot/distance of the cholesterol spot from the origin.

Gas Liquid Chromatography

GLC was carried out with a Barber-Colman Model 10 gas chromatograph having a hydrogen flame ionization detector. Pyrex glass columns (U-shaped, 6 ft long, 4 mm i.d.) were packed with 1% SE-30 (methyl silicone polymer), QF-1

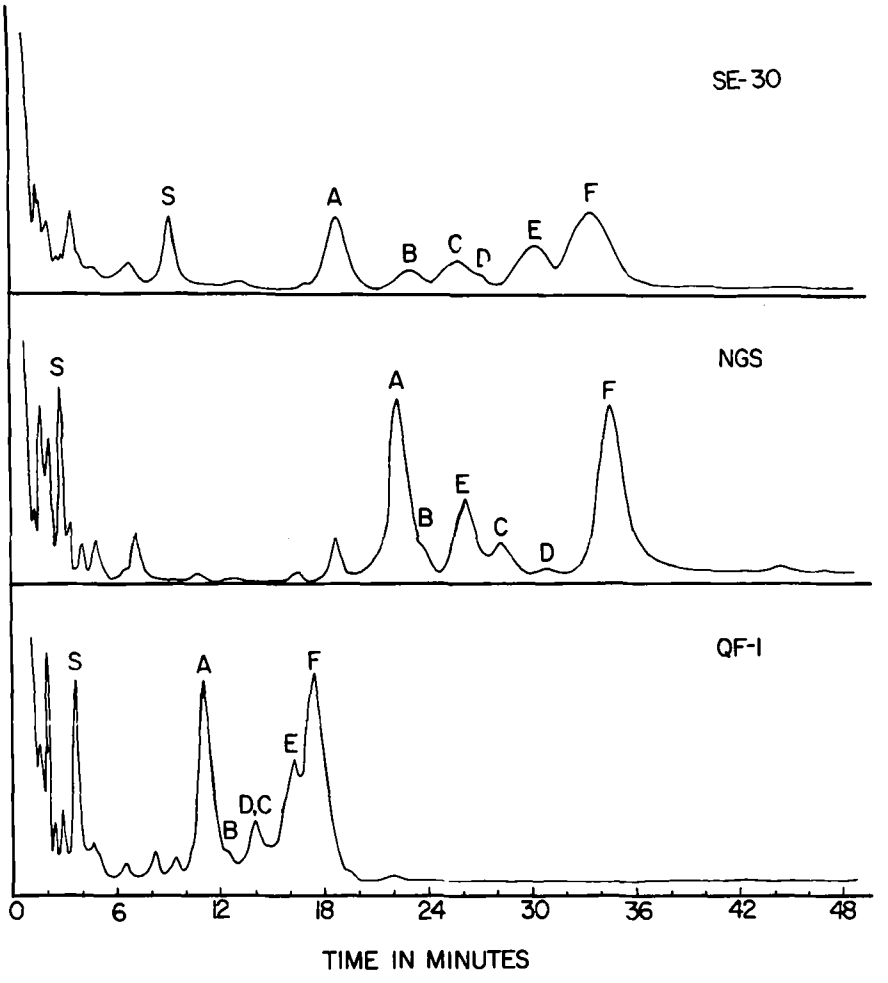

FIG. 4. Gas chromatograms of muscle unsaponifiables in zone 6 eluate from TLC plate (Fig. 1). Operating conditions: 6 ft x 4 mm i.d. column with 1% SE-30, NGS or QF-1 on Gas-Chrom Q, 80/100 mesh; column temperature, 211 C; 95 ml/min (for SE-30), 130 ml/min (for NGS) or 120 ml/min (for QF-1) N_2 carrier gas; hydrogen flame detector. Steroid peaks: A, cholesterol; B, Δ^8-methostenol; C, Δ^7-methostenol; D, dehydromethostenol; E, dihydrolanosterol; F, lanosterol; S, added cholestane as reference standard.

(fluorinated alkyl silicone polymer) and NGS (neopentyl glycol succinated polyester) on Gas Chrom Q, 80/100 mesh. The following operating conditions were used: nitrogen gas flow rate, 95 ml/min for SE-30, 120 ml/min for QF-1 and 130 ml/min for NGS; column temperature, 211 C; detector temperature, 255 C; flash evaporator temperature, 280 C. Samples of 1 to 2 μl were injected into the column with a 10 μl Hamilton syringe. Retention times were calculated relative to cholestane. The steroid number was calculated by the method of VandenHeuvel and Horning (11).

In quantitative studies with NGS column, the area of a peak was calculated by the triangulation method. Δ^8-Methostenol was used as the standard for the quantitative determination of dehydromethostenol.

Cholesterol Determination

Cholesterol content of muscle was determined by the method outlined by Tu et al.(3).

Proximate Analyses

Moisture and lipid contents of muscle and adipose tissue were determined by the methods outlined by Tu et al. (3).

RESULTS

Saponification

Unsaponifiable matter for this study was prepared by cold (25 C) rather than hot (refluxing) saponification to avoid chemical al-

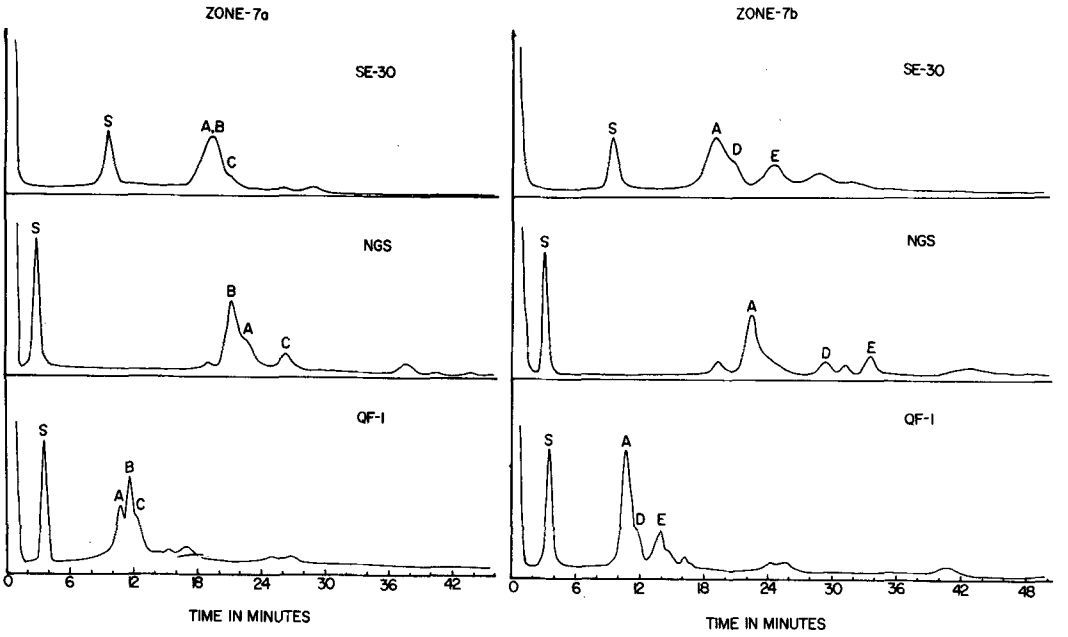

FIG. 5. Gas chromatograms of muscle unsaponifiables in zone 7a and 7b eluates from $AgNO_3$ TLC plate (Fig. 2). Operating conditions: 6 ft x 4 mm i.d. column with 1% SE-30, NGS or QF-1 on Gas-Chrom Q, 80/100 mesh; column temperature, 211 C; 95 ml/min (for SE-30), 130 ml/min (for NGS) or 120 ml/min (for QF-1) N_2 carrier gas; hydrogen flame detector. Steroid peaks: A, cholesterol; B, cholestanol; C, Δ^7-cholestenol; D, desmosterol; E, ergosterol; S, added cholestane as reference standard.

terations. Recently Chicoye et al. (12) demonstrated that 7-ketocholesterol in ethanolic KOH underwent extensive degradation at refluxing temperature, but not at 25 C. According to Menge et al. (13) the unidentified growth factor in the unsaponifiable fraction of egg yolk was destroyed when a refluxing temperature was used for saponification. In our study, comparison of TLC patterns for the unsaponifiable matter obtained by cold and hot saponification of muscle lipid revealed that some compounds were decomposed at the refluxing temperature. With the cold saponification procedure, the triglycerides were hydrolyzed completely, and thus did not interfere with TLC separation.

The average amount of unsaponifiable matter in two samples of bovine muscle (about 75% moisture and 5% lipid) was 95.5 mg/100 g of which about 60 mg was total cholesterol. In the case of adipose tissue (about 7.6% moisture and 90.7% lipid), the content of the unsaponifiable fraction was 400 mg/100 g. The total cholesterol concentration in the bovine adipose tissue was 88 mg/100 g.

Thin Layer Chromatography

Preparative TLC of the unsaponifiable matter from bovine muscle and adipose tissue was employed for separation into spot bands (each consisting of a series of spots across the preparative plate) of pure compounds or simple mixture of compounds. This preliminary TLC separation was essential for effective GLC resolution.

When silica gel chromatoplates for qualitative examination were developed with benzene-ethyl acetate (10:1), the unsaponifiable compounds were resolved into 10 spots for muscle and 8 spots for adipose tissue (Fig. 1). The R_c values for the spots on the chromatogram for adipose tissue unsaponifiables were similar to those for muscle unsaponifiables. Spot positions of a variety of authentic hydrocarbons, cholesterol esters and steroids with different degrees of polarity have been included in Figure 1 as reference compounds. Compounds in spot A at the top of the chromatogram were considered to be hydrocarbons. The R_c value and purple-brown spot color suggested that squalene was a major constituent. Spot B with a red hue (dull blue in UV) presumably consisted of cholesterol esters which were not hydrolyzed during cold saponification. This spot was not visible in the chromatogram with unsaponifiables after hot saponification. The brown-colored minor spots C and D, present only in

TABLE I

GLC Data of Steroids in TLC Zone 6 Eluate for Muscle (Fig. 4)

Steroids	Peak no.	SE-30 RRT[a] Experimental peak	SE-30 RRT[a] Reference peak	Steroid no.	NGS RRT Experimental peak	NGS RRT Reference peak	QF-1 RRT Experimental peak	QF-1 RRT Reference peak	Average steroid content (μg/100 g wet muscle)
Cholesterol	A	2.02	2.03	29.3	7.50	7.51	3.03	3.04	--
Δ^8-Methostenol	B	2.51	2.56	30.0	7.88	7.86	3.44	3.45	Trace
Δ^7-Methostenol	C	2.74	2.75	30.3	9.47	9.52	3.81	3.84	28.4
Dehydromethostenol[c]	D	2.92	(2.84)[b]	30.5	10.35	(10.30)	3.81	(3.75)	Trace
Dihydrolanosterol	E	3.21	3.25	30.9	8.80	8.86	4.43	4.48	55.4
Lanosterol	F	3.54	3.58	31.2	11.60	11.62	4.80	4.81	124.2

[a]Retention time relative to cholestane = 1.

[b]When a reference compound was not available, the relative retention time in parenthesis for the proposed structure was calculated as described in the text.

[c]4α-Methyl-$\Delta^{8,24}$-cholestadiene-3β-ol.

the chromatogram with muscle unsaponifiable matter, were located in the region where steroid esters and keto steroids would migrate. The moderately polar compound(s) in the brown-colored spot E was considered tentatively to be a keto steroid. After inspection of the R_c values and spot colors, spots F and G were considered to consist of a mixture of dihydrolanosterol and lanosterol and a mixture of Δ^7- and Δ^8-methostenol, respectively. Cholesterol and possibly minor amounts of structurally related steroids resided in the large spot H. The compounds in spots I and J were thought to be polyfunctional compounds, but not necessarily steroids.

As shown in Figure 1, each developed chromatoplate was divided into nine separate zones, each with at least one spot. For recovery of unsaponifiables (to be used for GLC) from each zone numbered 1 through 7, Rhodamine 6 G-silica gel preparative chromatoplates were used since the spot bands could be detected easily under UV light and the compounds could be eluted by chloroform without Rhodamine 6 G contamination. Since a polar solvent system (chloroform-methanol) was essential for eluting the polar compounds in zones 8 and 9, Rhodamine 6 G being soluble in this eluent was not added to the silica gel for TLC.

If biosynthesis of cholesterol occurs in muscle and adipose tissue, then steroids such as Δ^7-cholestenol and desmosterol would be expected in the unsaponifiable matter. These compounds, structurally similar to cholesterol, but differing in the number or location of double bonds, have R_c values (on silica gel chromatoplates) close to that of cholesterol (Fig. 1). The large amount of cholesterol in spot H could easily mask the spots of minor steroids in this spot region. To determine the presence of these cholesterol precursors, the spot band (zone 7) on preparative TLC plates was subdivided into two sections, 7i and 7ii. Presumably the minor steroids, if present, would be located as narrow bands at specific positions within the cholesterol band region and thus might be concentrated in either 7i or 7ii section. The eluate from each section was applied to preparative $AgNO_3$-silica gel chromatoplates which were subsequently developed with a chloroform-acetone (95:5) system. As shown on the analytical TLC plate in Figure 2, the reference compounds cholestanol (a), Δ^7-cholestenol (b), cholesterol (c), desmosterol (d) and ergosterol (e) were separated as distinct spots. The analytical chromatograms of the 7i and 7ii eluates for muscle (I) and adipose tissue (II) are presented in Figure 2. In zone 7a of the $AgNO_3$ plate, distinct spots of presumably cho-

lestanol and Δ^7-cholestenol (structural assignment based on their mobility and typical coloration) were obtained with the 7ii eluate, but the spot of cholestanol was barely visible in the 7i chromatograms. Under visible and UV lights, cholestanol and Δ^7-cholestenol spots on H_2SO_4-sprayed chromatoplates were yellowish-brown and pinkish-brown, respectively. Although tailing was noted in zone 7b of each of the chromatograms, distinct spots especially in the case of 7i eluate were in the desmosterol and ergosterol locations. Silica gel was scraped from zones 7a and 7b on preparative plates and the compounds were eluted with 50% ethanolic ammonia. Since ammonia complexes with silver ions (10), these metallic ions were not carried into hexane during reextraction. The eluates were examined by GLC.

Gas Liquid Chromatography

Eluates from each of the nine zones on preparative silica gel plates of muscle unsaponifiables were subjected to GLC with a nonselective SE-30 as the liquid phase. With adipose tissue unsaponifiables, zones other than 3, 4 and 5 (with relatively small or insignificant bands) were examined by GLC. The peak patterns in the gas chromatograms for muscle zones 1, 2 and 6 through 9 were similar to those for respective zones of adipose tissue unsaponifiables. Thus, to simplify the presentation, only the GLC patterns and retention values for muscle unsaponifiables have been included. The steroid numbers (SN) were calculated from the relative retention time (RRT) values with SE-30 to obtain a rough estimation of the number of carbon atoms in each peak compound and to aid in the identification of steroids. Common metabolic steroids have steroid numbers ranging between about 19 and 31 (11).

Figure 3 shows gas chromatograms of muscle unsaponifiables from zones 1, 2, 4, 5, 8 and 9. Zone 3 chromatogram has not been included since only very minor peaks were obtained. In the case of the zone 1 eluate, the chromatogram had one major peak along with numerous minor peaks. The RRT value of the major peak A was 1.10, the same value as that for the open-chain polyolefin, squalene (C_{30}). The presence of squalene in muscle and adipose tissue was further confirmed with QF-1 and NGS columns. The small peaks were considered to be hydrocarbons. The SN of 29.8 for the major peak A in zone 2 chromatogram was somewhat higher than that for cholesterol (29.3), but lower than that for cholesteryl acetate (30.8). Thus, peak A cannot be a cholesterol ester. The gas chromatogram in zone 4

TABLE II

GLC Data of Steroids in TLC Zone 6 Eluate for Adipose Tissue

Steroids	Peak no.	SE-30 RRT[a] Experimental peak	SE-30 RRT[a] Reference peak	Steroid no.	NGS RRT Experimental peak	NGS RRT Reference peak	QF-1 RRT Experimental peak	QF-1 RRT Reference peak	Average steroid content (μg/100 g wet tissue)
Cholesterol	A	2.00	2.03	29.3	7.45	7.51	3.03	3.04	---
Δ^8-Methostenol	B	2.50	2.56	30.0	7.90	7.86	3.45	3.45	Trace
Δ^7-Methostenol	C	2.76	2.75	30.3	9.45	9.52	3.82	3.84	48.8
Dehydromethostenol[c]	D	2.88	(2.84)[b]	30.5	10.34	(10.30)	3.82	(3.75)	Trace
Dihydrolanosterol	E	3.23	3.25	30.9	8.79	8.86	4.47	4.48	30.1
Lanosterol	F	3.57	3.58	31.2	11.65	11.62	4.84	4.81	414.0

[a]Retention time relative to cholestane = 1.

[b]When a reference compound was not available, the relative retention time in parenthesis for the proposed structure was calculated as described in the text.

[c]4α-Methyl-$\Delta^{8,24}$-cholestadiene-3β-ol.

TABLE III

GLC Data of Steroids in $AgNO_3$ TLC Zone 7a and 7b Eluates for Muscle (Fig. 5)

Steroids	Peak no.	SE-30 SE-30 RRT[a] Experimental peak	SE-30 SE-30 RRT[a] Reference peak	Steroid no.	NGS RRT Experimental peak	NGS RRT Reference peak	QF-1 RRT Experimental peak	QF-1 RRT Reference peak	Average steroid content (μg/100 g wet muscle)
Cholesterol	A	2.02	2.00	29.3	7.51	7.51	3.04	3.03	59,400
Cholestanol	B	2.05	2.05	29.4	7.06	7.06	3.31	3.31	51.0
Δ^7-Cholestenol	C	2.25	2.27	29.8	8.83	8.85	3.50	3.48	28.6
Desmosterol	D	2.20	2.21	29.6	9.80	9.80	3.29	3.28	114.0
Ergosterol	E	2.62	2.58	30.1	11.16	11.38	3.92	3.78	87.0

[a]Retention time relative to cholestane = 1.

had two major peaks with SN values of 24.2 for peak A and 26.2 for peak B. These peaks could not be identified. In our study, Δ^4-pregnene-3, 20-dione (progesterone) had an SN of 26.2, the same SN value calculated for peak B of zone 4. However, this polar compound moved only a short distance from the origin on the developed chromatoplate (Fig. 1). Only one large peak A was present in the chromatogram for zone 5. The SN of 24.6 for this peak compound is the same value as that for 4-androstene-3,16-dione (11). However, such a polar compound would not be expected to migrate to the spot E position on the TLC plate in zone 5 (Fig. 1). Two major chromatographic peaks, A and B, were obtained with zone 8 eluate whereas four major peaks were noted in the zone 9 chromatogram. The SN values, being 26.2 and below, indicate that if the peak compounds were steroids, they would be androgen, pregnane and estrane derivatives (11). No common hormonal steroids were found with GLC using the three liquid phases.

Since a large number of steroids were found in zones 6 and 7, detailed GLC data as related to structural identification is presented. Although only two distinct spots were present in muscle zone 6 on the chromatoplate (Fig. 1), six peaks with steroid numbers of 29.3 and above were in the gas chromatogram (Fig. 4). The high SN values were indicative of cholestane derivatives. The relative retention times (RRT) and SN of muscle unsaponifiable compounds in peaks A to F of the SE-30 gas chromatogram (Fig. 4) are tabulated in Table I. Peak A has been identified as cholesterol. The component in peak B had a RRT of 2.51 and SN of 30.0 which were almost identical to those for Δ^8-methostenol. On the basis of RRT, the compound in peak C was indistinguishable from Δ^7-methostenol. Dehydromethostenol was considered to be the component of peak D on the basis of similar RRT values. The RRT of dehydromethostenol, not being available as a reference compound, was estimated by multiplying the RRT of Δ^8-methostenol by a retention factor of 1.10 (for Δ^{24} functional group) obtained by the method outlined by Clayton et al. (14). The RRT values for peaks E and F were almost identical to those for dihydrolanosterol and lanosterol, respectively.

In order to further confirm the proposed structures for peak components in muscle zone 6, the zone 6 eluate was subjected to GLC with two selective liquid phases, QF-1 and NGS, for RRT comparisons. QF-1, a liquid phase with a high degree of stereoselective separating ability, was used to effectively separate hydroxy iso-

mers. As shown in Figure 4, the gas chromatographic resolution of steroids was poor when QF-1 was used as the liquid phase. Such a pattern is not surprising since QF-1 has no retention effects for carbon-carbon unsaturation (11). When NGS with carbon-carbon unsaturation selectivity was used as a column phase, the resolution of the peaks for zone 6 was much better. The RRT values of peak compounds separated on NGS and QF-1 are presented in Table I along with the RRT values for compounds with the assigned structures. Comparison of the RRT values with standards confirm the presence of cholesterol, Δ^7- and Δ^8-methostenol, dehydromethostenol, dihydrolanosterol and lanosterol in muscle zone 6. These methyl sterols were also detected in zone 6 for adipose tissue by GLC as shown in Table II. Williams and Pearson (2) reported the presence of lanosterol and cholesterol in pork fat.

When the eluate from muscle zone 7 was examined by GLC with SE-30, only one large, broad-spreading cholesterol peak was formed. Obviously, minor steroids would be masked if they were present. Thus, tentatively identified steroids in zones 7a and 7b were eluted from $AgNO_3$ chromatoplates (Fig. 2) for GLC analyses on SE-30, QF-1 and NGS. The Chromatograms are presented in Figure 5. For zone 7a, peaks B and C were identified, respectively, as cholestanol and Δ^7-cholestenol (from RRT comparisons in Table III) and peaks D and E for zone 7b were regarded to be, respectively, desmosterol and possibly ergosterol (as shown in Table III, experimental RRT values for QF-1 and NGS deviated slightly from those of ergosterol).

The quantitative estimation of steroids found in zones 6 and 7 for muscle and adipose tissue was carried out by GLC with NGS column which had relatively good resolving power. The steroid contents are presented in Tables I-IV. Although cholesterol is by far the major steroid in muscle and adipose tissue, significant amounts of Δ^7-methostenol, dihydrolanosterol, lanosterol, cholestanol, Δ^7-cholestenol, desmosterol and possibly ergosterol were present. Only trace amounts of Δ^8-methostenol and dehydromethostenol were found in tissues. The quantities of lanosterol and desmosterol were much higher in adipose tissue than in muscle.

DISCUSSION

In this investigation, TLC and GLC were employed as complementary techniques to separate and identify steroids in the unsaponifiable matter of bovine muscle and adipose

TABLE IV

GLC Data of Steroids in $AgNO_3$ TLC Zone 7a and 7b Eluates for Adipose Tissue

Steroids	Peak no.	SE-30 RRT[a] Experimental peak	SE-30 RRT[a] Reference peak	Steroid no.	NGS RRT Experimental peak	NGS RRT Reference peak	QF-1 RRT Experimental peak	QF-1 RRT Reference peak	Average steroid content (μg/100 g wet tissue)
Cholesterol	A	2.03	2.00	29.3	7.52	7.51	3.02	3.03	87,600
Cholestanol	B	2.06	2.05	29.4	7.09	7.06	3.32	3.31	49.2
Δ^7-Cholestenol	C	2.29	2.27	29.8	8.86	8.85	3.46	3.48	72.0
Desmosterol	D	2.23	2.21	29.6	9.80	9.81	3.28	3.28	368.8
Ergosterol	E	2.65	2.58	30.1	11.15	11.38	3.97	3.78	147.0

[a]Retention time relative to cholestane = 1.

tissue. Since investigators have shown that cholesterol can be biosynthesized in vitro from low-molecular weight precursors in skeletal muscle (8) and adipose tissue (9), a broad range of metabolic steroids would be expected to be present in these tissues.

Squalene, an open-chain C_{30} polyolefin, is a cholesterol precursor which undergoes enzymic polycyclization to lanosterol in the animal cell (15). Since Loud and Bucher (16) presented evidence that only a fraction of tissue squalene undergoes transformation to cholesterol, it is not surprising that a considerable amount of squalene was found by TLC (Fig. 1) and GLC (Fig. 3) in the unsaponifiable matter of bovine muscle and adipose tissue.

As shown in Tables I and II, lanosterol is one of the predominant steroids in the bovine tissues. This relatively high level of lanosterol may be attributed to a low rate of metabolic conversion (17,18). Dietschy and Siperstein (8) reported that a variety of extrahepatic tissues, not including muscle and adipose tissue, contained lanosterol. Lanosterol has been detected in the skin (14,19,20) and pork fat (2). The enzymic transformation of lanosterol to cholesterol includes the removal of the methyl groups at C-4 and C-14, the reduction of the isooctenyl side chain and the double bond transfer from the 8,9 to the 5,6 position (15). At present, the metabolic pathways are not clearcut. Avigan et al. (21) have suggested that the reduction of the 24,25 double bond may be one of the first enzymic modifications of lanosterol in liver.

As shown in Tables I and II, muscle and adipose tissue contain a considerable amount of dihydrolanosterol. Dihydrolanosterol has been found in skin (19,20) and in liver (22). Another possible mode of lanosterol alteration is the oxidation of the methyl groups (14,15,23). One of the metabolic products would be 4α-methyl-$\Delta^{8,24}$-cholestadien-3β-ol (dehydromethostanol) (14).

It is clear from Table I and II that only trace amounts of dehydromethostanol are present in bovine muscle and adipose tissue. This steroid and the Δ^7 analogue have been found in rat skin (14). Reduction of the 24,25 double bond of dehydromethostanol or removal of two methyl groups from dihydrolanosterol would lead to the formation of Δ^8-methostenol which could undergo a double bond shift to produce Δ^7-methostenol (15). Both Δ^7- and Δ^8-methostenols have been identified as constituents of a number of rat tissues (8). The Δ^8-isomer was first isolated from a preputial grand tumor of the mouse by Kandutsch and Russell (24). According to Wells and Neiderhiser (25), the Δ^7-isomer was isolated from rat feces. In our study, only Δ^7-methostenol was present at a relatively high concentration in muscle and adipose tissue but trace amounts of the Δ^8-isomer were also detected.

Demethylation of Δ^7-methostenol would result in the formation of Δ^7-cholestenol (15). In biosynthetic studies on cholesterol, Δ^7-cholestenol has been found in rat skin (14,20,21), and several other types of rat tissues (8). In our study, small quantities of Δ^7-cholestenol were estimated for bovine muscle and adipose tissue (Table III and IV). Δ^7-Cholestenol, as well as desmosterol, are considered to be immediate precursors of cholesterol (15).

Research has shown that desmosterol occurred in rat skin (14,26), chick embryo (27), rat liver (28), developing brain of the newborn rat (29), human, mouse and guinea pig (30), and nervous tissue of newborn rat (31). Avigan et al. (32) demonstrated that desmosterol accumulated in the liver to a high level when rats were fed triparanol, an inhibitor of cholesterol biosynthesis (33). In our study, approximately 114 μg/100 g of desmosterol was determined for bovine muscle while about three times this amount was found in the adipose tissue.

Cholestanol and possibly ergosterol were identified as sterols in bovine tissues. Cholestanol is distributed widely in mammalian tissue (34-36). With liver homogenate of the rat and guinea pig, Shefer et al. (37) showed that cholesterol as well as mevalonate was converted into cholestanol. Dietschy and Siperstein (8) indicated that cholestanol was biosynthesized from acetate in a variety of rat tissues. According to Rosenfeld et al. (38), cholesterol injected into a human was converted to cholestanol via a 3-ketonic intermediate. Shefer et al. (39) reported that conversion of 5α-cholestane-3-one to cholestanol in rat liver was catalyzed by 3-hydroxysteroid dehydrogenase. Mammalian tissues are apparently capable of synthesizing cholestanol using acetate as well as cholesterol. However, the physiological function of cholestanol in tissue is still little known. So far, ergosterol has not been isolated from animal tissues, but Pennock et al. (40) reported that avian yolk contained a small amount of this sterol.

Several investigators have demonstrated the enzymic transformation of hormonal steroids in muscle in vitro. Sweat and Bryson (5) reported the enzymic oxidation and reduction of cortisol in bovine skeletal muscle. According to Lipman et al. (6), hydroxylation of cortisol at C-6 occurred during incubation with human skeletal

muscle. The metabolism of Δ^4-androstene-3, 17-dione incubated with rabbit muscle was demonstrated by Kochakian and Stidworthy (41) and Thomas and Dorfman (7). With the aid of TLC and GLC, none of the common hormonal steroids were detected in our investigation.

With the exception of rat skin, few studies have been directed toward steroid composition of normal intact tissues. Cholesterol was reported to be the only steroid found in serum, liver, aorta and brain of the untreated rat (29). Similar results are also demonstrated by Horlick (20). Apparently, cholesterol is the only major steroid in mammalian tissues and other steroids, if present, probably occur in very small amounts. The results of this study provide support for this presumption.

ACKNOWLEDGMENT

This study was supported by a grant from the National Live Stock and Meat Board.

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[Received October 3, 1968]

Cholesterol Content of Human Parotid Saliva

GERT M. JACOBSOHN and **RAFAEL J. MONTIEL**[1] Department of Biological Chemistry, Hahnemann Medical College, Philadelphia, Pennsylvania 19102

ABSTRACT

Free and esterifed cholesterol were determined in parotid fluid collected from 19 individuals by encapsulation of Stenson's duct in the oral cavity. Free cholesterol content averaged 16.7 ± 7.1 (SD) μg/100 ml and cholesterol esters averaged 13.2 ± 8.0 (SD) μg/100 ml. Two assays are described for the measurement of cholesterol in parotid saliva. Lipophilic material was extracted with ether-ethanol (3:1). In assay 1, the solid residue of the extract was separated into its components by thin layer chromatography. Substances on the chromatogram were made to fluoresce and the spots were photographed. Cholesterol was determined by densitometry of its black image on film. In assay 2, the ether-ethanol residue was subjected to quantitative gas chromatography. Both methods yield similar data.

INTRODUCTION

Parotid fluid is a clear, watery secretion which together with submaxillary and sublingual fluids constitutes whole saliva. The total volume of saliva is generally estimated at around 1500 ml per day. This is a considerable amount and should lend itself to investigations on the nature of its constituents as a test for biochemical dynamics throughout the organism. The ease of saliva collection without causing discomfort to the subject and without stress makes it possible to collect serial samples over a prolonged period of time. Development of self-positioning devices for collection of parotid fluid by Shannon and Terry (1) allows a subject to collect his own saliva for monitoring sterol levels during times when professional personnel may not be available.

Information in the literature on the sterol content of saliva is scanty. Thirty years ago, Krasnow (2) reported a range of 2.4 to 50 mg/100 ml of whole saliva. This is an appreciable quantity which may be of import to cholesterol metabolism of the entire organism. However, the observed values are almost certainly too high since cholesterol was determined by a color test performed on crude extracts. Saliva used in that study could have been contaminated with extra salivary digestive secretions and with food rests. It was therefore decided to reexamine this problem with modern, highly sensitive techniques. Analysis by gas chromatography was sensitive to 40 ng of cholesterol.

EXPERIMENTAL PROCEDURES

Saliva Collection and Extraction

Parotid fluid was collected from apparently healthy volunteers by means of a double-lumen teflon cup of the type described by Carlson and Crittenden (3) and by Curby (4). The device consists of a disc of 21 mm in diameter with two concentric chambers. The outer one is connected by thin polyethylene tubing to a syringe for applying suction and the inner one leads to a collecting vessel with similar tubing. The inner chamber was placed directly over the opening of Stenson's duct in the oral cavity and a vacuum was produced by aspiration of the syringe to keep the device in place. Flow of saliva was stimulated by Life Savers or lemon drops. In some cases two cups were used simultaneously, one over each duct. The collection period lasted up to 1/2 hr and started no less than 2 hr postprandially, usually after breakfast, without previous smoking and after rinsing the mouth with water. A maximum of 50 ml of saliva per duct could be collected during the time period alloted.

For free cholesterol determinations, each sample of 10 ml of freshly collected saliva was added to 50 ml of a mixture of 3 parts of ethyl ether and 1 part of ethanol. The mixture was shaken vigorously and was then separated. The organic phase was taken to dryness under nitrogen in a water bath at a temperature not exceeding 50 C. The residues were transferred with ether to micro test tubes. These were produced from glass tubing 3.4 mm I.D. and 3.5 cm long and were supplied with a tooled opening to accept silicone rubber septa (Applied Science Labs., State College, Pa., Cat. No. W-10). A portion of the residues were assayed by photogrammetry or by gas chromatography.

To determine total cholesterol, samples were extracted as before with ether-ethanol. After

[1]On leave of absence from the Facultad de Medicina, Universidad del Zulia, Maracaibo, Venezuela.

evaporation of the volatile phase, 5 ml of 6% of potassium hydroxide in ethanol was added to the residues, and the mixture was heated in a boiling water bath for 30 min. After cooling to room temperature, the mixture was treated with 5 ml of water and extracted 3 times with 10 ml portions of ether. The pooled ether extracts were washed 3 times with 10 ml of water and evaporated. The residues were transferred to micro test tubes and assayed. Esterified cholesterol was calculated as total minus free cholesterol. All assays were done in duplicate or triplicate. In 3 instances not enough saliva was available for determining the esterified fraction.

Photogrammetric Assay

The procedure has been described previously for the determination of estrogenic steroids (5,6). The sterol residues were dissolved in 0.2 ml of ethanol and 10 and 20 μl samples were applied to lanes on thin layer chromatography plates. The plates had been prepared from silica gel H (E. Merck AG, Darmstadt) to a uniform thickness of 0.25 mm. The lanes were 1.5 cm wide and a maximum of 12 samples could be applied per plate. Samples of standard cholesterol of 0.15, 0.30, 0.60 and 0.90 μg were applied to 4 lanes. After drying briefly at room temperature, the plates were placed inside a solvent-saturated chromatography tank containing a mixture of 8.5% of ethanol in methylene chloride. When the solvent had ascended to a height of 15 cm from the origin, the plates were withdrawn and dried in air. They were sprayed with a 50% aqueous solution of sulfuric acid and heated to 110 C for 8 to 10 min. Cholesterol became visible as purplish-blue spots which fluoresced yellow-green under ultraviolet light. In the system described, cholesterol has an R_f value of 0.57.

The sprayed chromatograms were removed to a dark room in which the sole source of illumination was afforded by an ultraviolet lamp with maximum intensity at 360 mμ. Photographs were taken on Kodak Royal Pan sheet film of size 8 X 10 in. for an exposure time of 2 min at a lens opening of f/8.0 and through an orange Wratten filter number 16. The film was developed in Polydol diluted from stock solution with twice its volume of water. Film was put through a stop bath, fixed, thoroughly washed and dried. The original lanes which had been engraved on thin layer plates could be seen lightly on the developed film. The lanes were cut and the dark spots corresponding to cholesterol were evaluated by means of a recording densitometer.

Gas Chromatographic Assay

Sterol residues from saliva were dissolved in 50 μl of ethanol and portions of 2 to 5 μl, depending on the concentration, were injected into the column. The gas chromatograph was a Glowall Model 320 fitted with a hydrogen flame ionization detector at an emf of 350 v. The temperature of the detector oven was 260 C, the column oven was set at 250 C and the flash heater registered 260 C. The gas phase was argon at a flow rate of 30 ml/min. The column was made up of Gas Chrom Q (100-120 mesh) coated with 3% by weight of OV-1 (Applied Science Labs.) and had a length of 180 cm and a diameter of 3.4 mm. Cholesterol emerged with a peak at 10.6 min. No other peaks were present in the vicinity of the cholesterol peak. Linearity of response, measured by planimetry of peak areas, was obtained when cholesterol was injected in amounts of 0.04 to 0.5 μg. The unknown samples fell into this range.

RESULTS

In Table I are shown data for 19 individual samples of parotid fluid. The range of values for free cholesterol was 5.9 to 33 μg/100 ml and the esterified fraction ranged from 2.2 to 24.7 μg/100 ml. In some instances the esterified fraction was not determined due to a lack of sufficient saliva. Values shown are uncorrected for losses of procedure but it is believed that these are minimal due to the much higher solubility of cholesterol in ether-ethanol (3:1) or ether compared to water. Assays by both gas chromatography and photogrammetry gave the same results and were used interchangeably.

An example of a densitometric tracing of cholesterol isolated from saliva and assayed by photogrammetry is shown in Figure 1. The small, sharp peak corresponds to a line drawn on the film strip for reference. The developed films showed a clear background. Besides the cholesterol spot, a second much weaker spot appeared with an R_f value of 0.17. It was present in most samples examined but has not been identified thus far. Less polar material moved close to the solvent front where it formed a spot. This spot includes cholesterol esters since authentic cholesterol palmitate moved to the same region on the thin layer plate. It was not possible to measure both free and esterified cholesterol on the same plate due to interference of material at the solvent front with the esterified portion. When the area at the solvent front of a thin layer chromatogram of a previously unhydrolyzed saliva extract was eluted, hydrolyzed with 6% potassium hy-

TABLE I

Cholesterol Content of Parotid Fluid (μg/100 ml)

Sex	Age	Free cholesterol	Esterified cholesterol
F	22	13.2	5.8
F	18	5.9	
F	18	10.3	24.7
F	18	15.0	5.4
F	19	20.0	20.0
F	18	23.0	22.0
F	24	15.2	
F	19	15.5	2.2
F	19	11.0	
M	26	20.0	13.5
M	36	16.5	23.0
M	37	9.0	23.0
M	38	18.5	8.0
M	27	33.0	4.0
M	41	12.9	5.6
M	23	31.5	6.0
M	41	9.0	12.0
M	23	15.4	15.9
M	28	21.5	19.5
Average		16.7	13.2
Standard deviation		± 7.1	± 8.0

droxide, extracted and chromatographed again, a spot identical to cholesterol standard appeared. Repetition of the hydrolysis on material at the solvent front did not liberate additional sterol.

The identity of salivary cholesterol was ascertained by comparison to authentic material. Standard cholesterol and the main components from salivary extracts ran the same distances on thin layer chromatograms. The color of the spots in visible and in ultraviolet lights after spraying with sulfuric acid was the same for standard cholesterol and salivary material, as were the fading characteristics over a 24 hr period. Gas chromatograms of eluates from thin layer plates in the region of cholesterol coincided in retention time with authentic sterol. The system consisted of 0.8% of SE-52 coated on Gas Chrom Q (7). An augmentation curve in which standard cholesterol was added to a salivary eluate so as to double the amount of sterol, showed a symmetrical increase in the peak corresponding to cholesterol. Three small, broad bands, estimated in total as no more than 8% of the cholesterol peak, appeared after 19 min. The gas chromatographic system does not separate cholestanol from cholesterol and it is possible that a small portion of this sterol may be present.

Gas chromatography of saponified saliva extracts on a column of 3% OV-1 on Gas Chrom Q showed a main peak with the retention time of cholesterol. A smaller peak with an R_t of 0.52 (cholesterol =1) was present in all

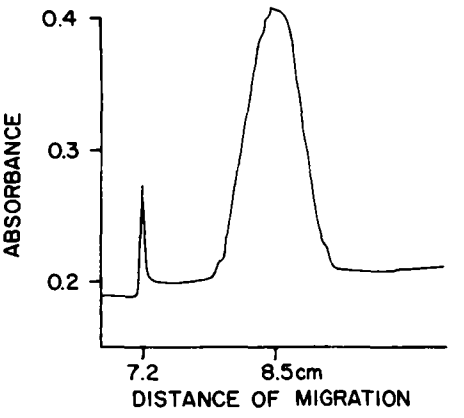

FIG. 1. Densitometric tracing of typical salivary cholesterol spot reproduced on film.

samples. Two minute peaks occurred at R_t of 0.64 and 0.83 and a small, broad band could be seen with an R_t of 1.44. Unhydrolyzed extracts also showed the same peaks but with less intensity. The smaller peaks were frequently not detectable.

DISCUSSION

The average cholesterol content of parotid saliva from 19 samples of healthy individuals was 16.7 ± 7.1 (SD) μg/100 ml. Similarly, cholesterol esters amounted to 13.2 ± 8.0 (SD) μg/100 ml. The relative ratios of free to esterified cholesterol cannot be considered significant until a larger number of samples has been examined. Nor is it possible to state what portion of cholesterol originates from plasma and what portion is synthesized by the gland itself. A transfer of cholesterol from plasma is favored as a means of raising cholesterol concentration in saliva. Precedence exists for the transfer of numerous substances, including certain steroids which have been studied thus far. Katz and Shannon (8) observed that corticosteroid levels in parotid fluid are in direct correspondence with the plasma concentration. They found further (op. cit.) that a portion of peripherally injected 4-androsten-3, 17-dione and 17β-estradiol can be recovered from parotid fluid. By this example we expect that most, if not all, of the salivary cholesterol derives from plasma.

Parotid fluid has not been assayed previously for quantitative cholesterol content. Mandel and Ellison (9) report presence of free and esterified cholesterol in parotid and submaxillary saliva by an unspecified identification method. Dirksen (10), working with extracts from whole and parotid saliva, identified cho-

lesterol and its esters by paper chromatography. Ranges of total cholesterol concentration of 2.0 to 9.0 mg/100 ml and of 2.4 to 50 mg/100 ml of whole saliva have been reported by Krasnow and Oblatt (2,11). These values appear excessively high, particularly since no precautions were taken to collect saliva without contaminants. The data were presented as total Lieberman-Burchard positive substances, without exclusion of nonsterol impurities. The assay was performed by concentrating saliva collected by spitting, to one quarter of its original volume, mixing with plaster of Paris and extracting with chloroform. The color test was performed on the crude residue after solvent evaporation.

The cholesterol in parotid fluid exists in soluble form, possibly as a complex with protein. This is brought out by the low solubility of cholesterol in water. Saad and Higuchi (12) found that cholesterol dissolves to the extent of 2.6 μg/100 ml of water at 30 C. It can be assumed that protein binding is responsible for the additional quantity of cholesterol in saliva. Human salivary mucin does not contain measurable cholesterol as an integral component (13).

The possibility exists that the collected saliva was contaminated with sloughed off cells of the area of contact between collecting cup and oral surface. The likelihood of an irritation of the mucosa was considered low because of the smoothness of the teflon collecting devices. When saliva samples were examined by phase contrast microscopy, an occassional squamous cell could be observed. The maximum number of cells that could be present was calculated at less than 50/ml. This is too low to be of concern in this work. Other cells could not be found. The Life Savers or lemon drops used to stimulate the flow of saliva did not contain any detectable sterols, as tested on extracts by thin layer and gas chromatography.

ACKNOWLEDGMENTS

Warren Chernick of this institution contributed a sample of the parotid fluid collecting cup and gave instruction on its use; Ihor Shpernal provided technical assistance. The Ministry of Health of Venezuela provided travel funds and other support; the Heart Association of Southeastern Pennsylvania gave financial assistance.

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[Received November 19, 1968]

Variation in Plasma Lipids With Age and Sex in a Hypertriglyceridemic Rat

PAUL H. LENZ, Department of Biology, Fairleigh Dickinson University, Madison, New Jersey 07940, and **ALAN I. FLEISCHMAN**, Atherosclerosis Research Group, St. Vincent's Hospital, Montclair, New Jersey 07042

ABSTRACT

A substrain of the Long Evans rat having the characteristics of a common human lipid disorder, hypertriglyceridemia, is described. These rats were found to be hypercholesterolemic, hyperphospholipidemic and normotriglyceridemic during the first month of life when feeding predominantly on the higher lipid, lower carbohydrate content of their mothers' milk. After weaning (22 days of age), when a typical higher carbohydrate and lower fat containing commercial laboratory feed was substituted for mother's milk, plasma cholesterol and phospholipid levels declined and triglyceride levels significantly increased. Electrophoretic analysis of lipoproteins revealed the presence of a pre-β fraction and the absence of chylomicra, indicating that this rat is a type IV according to the Fredrickson classification system. Glucose tolerance curves remained elevated for a longer period of time and returned to normal more slowly in 1 year old compared to 2 month old males, indicating progressive alterations in glucose metabolism common in a Fredrickson type IV. After weaning, plasma phospholipid, cholesterol and triglyceride levels were significantly higher and body weight significantly lower in the female than in the male. Studies on hematocrit and hemoglobin variations with respect to age in both sexes indicated that the observed plasma lipid changes could not be attributed to hemoconcentration.

INTRODUCTION

In the study of the control of common human lipid disorders, the development of model animal systems is important. In a previous study with rats from an inbred colony of the Long Evans strain, it was noted that the rats were naturally normocholesterolemic but significantly hypertriglyceridemic (1). In order for this substrain to be used in studying means for controlling hypertriglyceridemia, it appeared necessary to determine whether the hypertriglyceridemia was present for a significant period in the rat's life cycle or whether it was a transient effect. In addition, the specific type of dyslipoproteinemia accompanying the hypertriglyceridemia required characterization. The results of some studies on the variation in blood lipids with age and sex and a characterization of the lipoproteinemia are presented in this report.

MATERIALS AND METHODS

In these studies rats from an inbred hypertriglyceridemic colony of the Long Evans strain were used. They were weaned at 23 days of age and maintained on a commercial rat diet (Purina Laboratory chow) and tap water ad libitum. For each time period studied, a minimum of 10 rats of each sex were used. With rats less than 23 days of age, it was necessary to pool the blood of two (15 day old) to six (3 day old) rats in order to obtain sufficient sample for analysis, and thus the data for these groups represent correspondingly more rats. After an 18 hr fast, the rats were anesthetized with sodium pentobarbital, 50 mg/kg body weight, and exsanguinated via the dorsal aorta. The 3 and 15 day old rats were bled by decapitation. Blood was collected in tubes containing 9 mg disodium ethylenediaminetetraacetic acid; the plasma was separated immediately and assayed.

Plasma cholesterol, triglycerides and phospholipids were determined by previously described automated methods (2). Glucose tolerance tests were performed by gavage feeding 2 g glucose/kg body weight and determining the blood true glucose levels at half hour intervals for the first 2 hrs and then at hourly intervals to 9 hrs. Blood samples for true glucose analysis were taken from the tail in 50 μl heparinized capillary tubes, centrifuged, and the plasma assayed by an ultra-micro modification of the glucose oxidase-peroxidase method (3-6). Plasma lipoproteins were determined by electrophoresis on acetate for 45 min at 350 v in tris-barbital sodium buffer, pH 8.8, ionic strength 0.028 which was 0.001 M in disodium ethylenediaminetetraacetate (7). The lipoproteins were visualized by oxidation over barium peroxide-sulfuric acid and staining with Shiff's stain (8). Quantitation was accomplished by photodensitometry without preliminary

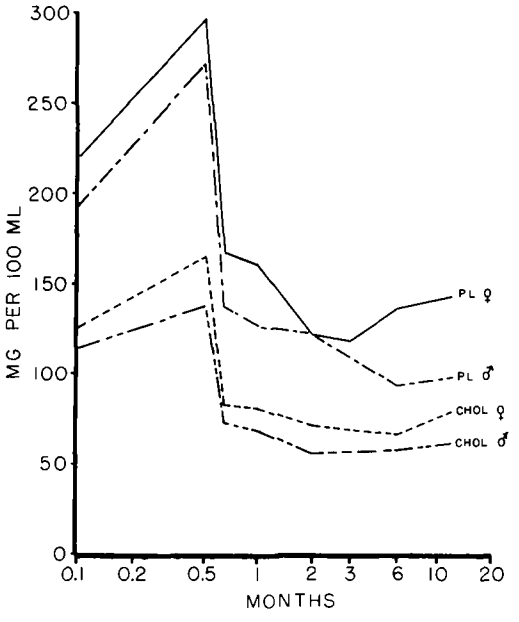

FIG. 1. Variation in plasma cholesterol and phospholipid with age and sex, PL, phospholipids; Chol, cholesterol.

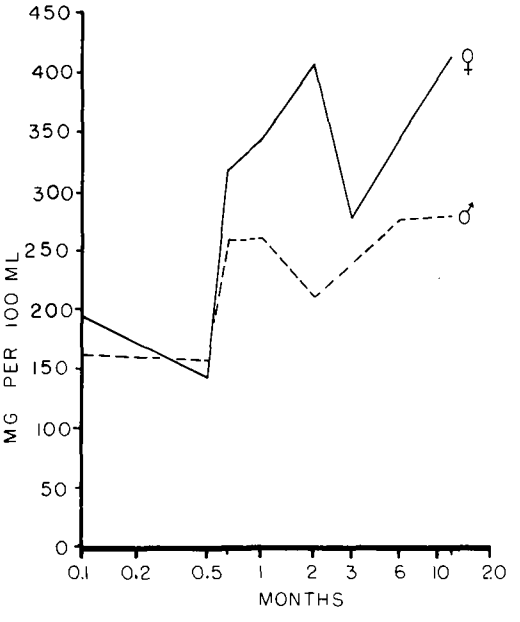

FIG. 2. Variation in plasma triglycerides with age and sex.

clearing. Studies with triglyceride loaded rats had shown that this method was capable of resolving lipoproteins into four bands with the mobilities of chylomicra, β-lipoproteins, pre-β-lipoproteins and α-lipoproteins. In some cases trail substance was noted. Hemoglobin was determined by the Unopette modification of the cyanmethemoglobin technique (9).

Analyses for changes were performed on the data by use of appropriate estimates of error and Student t-tests (10).

RESULTS

The variation of plasma cholesterol and phospholipids with respect to age and sex is shown in Figure 1. At 3 days of age plasma phospholipids were 220 ± 10.6 and 192 ± 13.6 mg/100 ml in females and males respectively. A significant rise occurred by 15 days, $P < 0.01$, in both sexes. The levels progressively decreased in both sexes until 60 days of age, $P < 0.01$. The male then remained essentially unchanged for the remainder of the 1 year study period. The female exhibited a small but significant rise in phospholipids after reaching puberty, $P < 0.01$, and this level was maintained to the end of the study period. The phospholipids were significantly higher in the female compared to the male from puberty to the end of the study period.

The pattern of plasma cholesterol changes was essentially the same as that for the phospholipids in both sexes. A significant rise over the 3 day level occurred by 15 days, $P < 0.01$, followed by a progressive significant drop until 60 days of age, $P < 0.01$. No significant further changes occurred for the remainder of the 1 year study period. The cholesterol level in the female was significantly higher than in the male, $P < 0.05$, after puberty.

The variations in plasma triglycerides with respect to age and sex are depicted in Figure 2. At 3 days of age the levels were 192 ± 28.7 and 161 ± 15.5 mg/100 ml in females and males respectively. No significant change occurred by 15 days of age. By 23 days a very significant rise occurred in both sexes, $P < 0.01$. A peak value of 260 ± 10.2 mg/100 ml was attained in the male at 30 days of age. This was followed by a small, not statistically significant, drop at 2 months after which the level returned to and remained at the original peak level for the remainder of the 1 year study. The female reached a peak of 408 ± 54.6 mg/100 ml at 2 months of age followed by a significant decline at 3 months, $P < 0.05$. The triglyceride levels then rose progressively for the remainder of the study period and reached the initial peak level by 1 year. At all time periods beyond 23 days of age, the triglyceride levels in the female were significantly higher than in the male, $P < 0.05$.

Since the triglycerides were elevated, it seemed desirable to determine the transport vehicle for the triglycerides. Electrophoretic

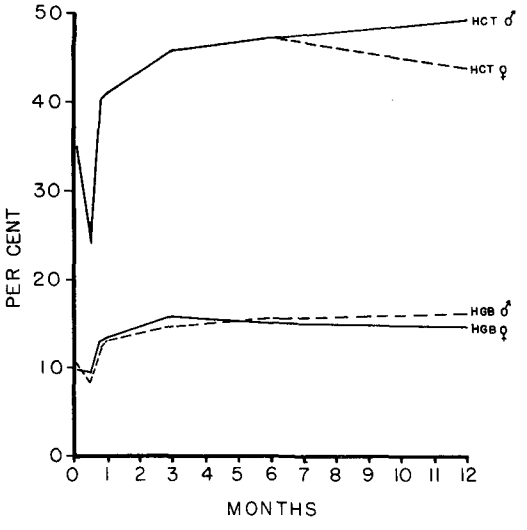

FIG. 3. Variation in hematocrit and hemaglobin with age and sex. HCT, hematocrit; HGB, hemaglobin.

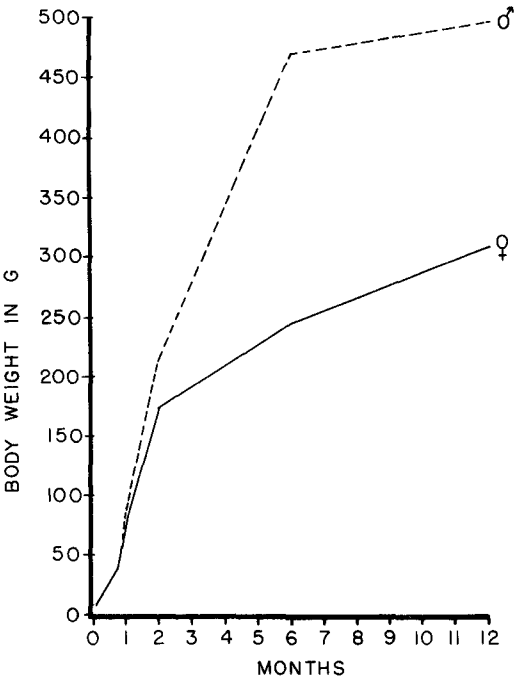

FIG. 4. Variation in body weight with age and sex.

analysis of the lipoproteins at both 2 months and 1 year of age indicated the percentage of the total stain absorbed by the lipoproteins was distributed as follows: chylomicra absent, 14.2 ± 3.1% β-lipoprotein, 22.9 ± 5.3% pre-β-lipoprotein and 62.9 ± 6.1% α-lipoprotein. No significant age or sex variation was observed. This would indicate that this rat is a type IV phenotype according to the Fredrickson classification (11-12).

Among the abnormalities that could accompany a Fredrickson phenotype IV are progressive alterations in glucose metabolism (13). In Table I are depicted the mean glucose tolerance curves at 2 months and 1 year of age, in the male. At 2 months of age, an essentially normal glucose tolerance curve was obtained in which the maximum glucose concentration was attained at 30 min followed immediately by a drop in blood glucose to normal levels by 3 hr. In the 1 year old animals there was a significant delay in the start of the drop, $P < 0.01$; the peak level remained for 1.5 hr. The drop in blood glucose was much slower and the level did not return to normal until the eighth hour. No significant difference was noted in the glucose tolerance test between males and females at 1 year of age.

In order to determine whether at any time period the serum lipid values were falsely elevated due to hemoconcentration, hemoglobin and hematocrit values were determined (Fig. 3). The hemoglobin and hematocrit levels were not related to sex. The initial dip in hematocrit at 15 days followed by a rise until 2 months of age is essentially similar to that found in humans (17). The decline was significant at $P < 0.01$ as was the subsequent rise.

In Figure 4 are shown the variations in body weight with age and sex. The body weight changes are essentially what might be expected, with the male significantly heavier than the female after 2 months of age, $P < 0.01$.

DISCUSSION

It was noted that the plasma triglycerides were significantly elevated after 23 days of age in both sexes and remained elevated to the end of the 1 year study period (Fig. 2). During the suckling period, when the rats were subsisting on the higher fat, lower carbohydrate content of mothers' milk, plasma triglyceride levels were significantly lower. At 23 days of age, when the plasma triglycerides began to rise, the rats had been weaned and were subsisting on a commercial laboratory chow containing 4.3% fat, 50.8% carbohydrate and 23.4% protein. In view of the elevated plasma triglycerides, the plasma lipoprotein patterns were studied in order to differentiate between the three possible Fredrickson phenotypes associated with hypertriglyceridemia (11,12). The plasma lipoprotein patterns showed a higher pre-β-lipoprotein content indicative of possible carbohydrate induced hypertriglyceridemia. The absence of definitive chylomicra bands

TABLE I

Glucose Tolerance Curves for Male Hypertriglyceridemic Rats at 2 Months and 1 Year of Age

Time after glucose Administration, hr	Age	
	2 Months 8 Rats Plasma glucose	1 Year 9 Rats Mg/100 Ml
0	86 ± 8.8[a]	104 ± 4.4
0.5	176 ± 11.0	169 ± 8.1
1.0	163 ± 7.2	168 ± 6.9
1.5		167 ± 6.6
2.0	125 ± 4.6	150 ± 5.8[b]
3.0	103 ± 9.6	130 ± 8.1[c]
4.0	93 ± 6.2	122 ± 4.4[b]
5.0	108 ± 4.1	126 ± 4.9[c]
6.0		128 ± 6.0
7.0	96 ± 5.1	127 ± 5.8[b]
8.0		102 ± 6.1
9.0	88 ± 7.1	107 ± 3.5[c]

[a]Mean ± standard deviation.

[b]$P < 0.01$ comparing 1 year with 2 months.

[c]$P < 0.05$ comparing 1 year with 2 months.

negated the possibility of hypertriglyceridemia being due to a deficiency in lipoprotein lipase (Fredrickson phenotype I or V). The data would seem to indicate that this rat substrain is an experimental model of one of the more common human lipid disorders, Fredrickson phenotype IV.

Several pathological situations are known to be common in the human Fredrickson type IV individual, i.e., diabetes, hypothyroidism and cirrhosis of the liver (11-13). In this study, glucose tolerance curves showed a progressive increase ($P < 0.01$) in the time required for plasma glucose to return to fasting levels, indicating progressive alterations in glucose metabolism (Fig. 3).

As in the human, this strain of rat seems to be responsive to the carbohydrate-to-fat ratio in the diet. Thus, during the suckling period where the ratio of carbohydrate to fat in the milk is approximately 0:2 (15), both the serum cholesterol and phospholipids are significantly higher and the triglycerides are significantly lower than after weaning when the rats are consuming a diet containing a carbohydrate-to-fat ratio of approximately 11:1.

A rat substrain model of possible carbohydrate inducible hypertriglyceridemia could be a valuable tool in evaluating ways of controlling this common human lipid disorder. Studies in this direction are now in progress.

ACKNOWLEDGMENTS

Supported in part by Grant H-5905, National Heart Institute, and Research Grants 81.30 and 81.35, Fairleigh Dickinson University. H. W. Gerarde, of Becton Dickinson and Company, Rutherford, N.J. contributed Unopettes used in the ultra-micro determination of blood true glucose and hemoglobin. Marvin Fleischman prepared the drawings.

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[Received December 20, 1968]

Regulation of Hepatic Carbohydrate Metabolism by FFA and Acetyl-CoA: Sequential Feedback Inhibition

G. WEBER, M. A. LEA, and **NANCY B. STAMM,** Department of Pharmacology, Indiana University School of Medicine, Indianapolis, Indiana 46202

ABSTRACT

In recent years experiments in slices, perfused liver and in the whole animal demonstrated that liver carbohydrate metabolism can be controlled by free fatty acids (FFA). Our investigations suggest that FFA and acetyl-CoA might provide regulation of the rate and direction of opposing pathways of hepatic glycolysis and gluconeogenesis by controlling the activity of key enzyme systems. Long and short chain FFA were observed to selectively inhibit the key enzymes of glucose catabolism, glucokinase, hexokinase, phosphofructokinase and pyruvate kinase. The long chain FFA were inhibitors two magnitudes stronger than octanoate. The FFA were also able to inhibit lactate production in a fortified supernatant fluid system. Acetyl-CoA inhibited hepatic glucokinase and pyruvate kinase but did not affect liver hexokinase, phosphofructokinase, lactate dehydrogenase, glucose-6-phosphatase or fructose-1,6-diphosphatase. The inhibition of glucokinase and pyruvate kinase was dependent on the dose and preincubation time with acetyl-CoA. The acetyl-CoA is the end product of the degradation of FFA which in turn is an end product of glucose catabolism; therefore, the inhibition of the three key glycolytic enzymes by FFA and the subsequent reinforcement of the inhibition of glucokinase and pyruvate kinase may be called sequential feedback inhibition. The regulatory role of phosphoenolpyruvate, NADH, ATP, alanine and oxaloacetate in the control of hepatic carbohydrate metabolism is discussed.

INTRODUCTION

Evidence has accumulated in recent years that hepatic carbohydrate metabolism can be regulated by lipid metabolites as indicated by experiments in liver slices, in perfused liver and in the whole animal (1-7). Our investigations suggest that along with several other signal molecules the free fatty acids (FFA) (8-10) and acetyl-coenzyme A (acetyl-CoA) (11) provide regulation of the rate and direction of the opposing pathways of hepatic gluconeogenesis and glycolysis by controlling the activities of key enzyme systems (12). Figure 1 illustrates the two opposing pathways and compares the activities of the key gluconeogenic enzymes (glucose-6-phosphatase, fructose-1,6-diphosphatase, phosphoenolpyruvate carboxykinase and pyruvate carboxylase) and the key glycolytic enzymes (glucokinase, phosphofructokinase and pyruvate kinase). Consideration of the activities of the opposing enzymes suggests that mechanisms must exist which are capable of rapidly inhibiting the activities of pyruvate kinase and the other two key glycolytic enzymes, glucokinase and phosphofructokinase. The inhibition of pyruvate kinase is especially necessary, since such a mechanism would facilitate gluconeogenesis and, through decreasing glycolysis, prevent recycling (8,10).

An alteration in the amount of the glycolytic enzymes and of the opposing gluconeogenic enzymes may provide a change in the ratio which would result in an alteration in the overall potential and could change the direction of the opposing pathways. Indeed, under various gluconeogenic conditions there is an increase in the biosynthesis of gluconeogenic

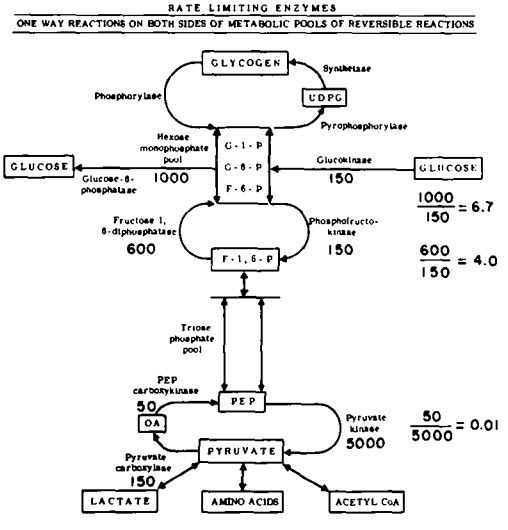

FIG. 1. Comparison of the activities of key gluconeogenic and glycolytic enzymes. Activities are expressed in μmoles of substrate metabolized per gram wet weight/hr at 37 C.

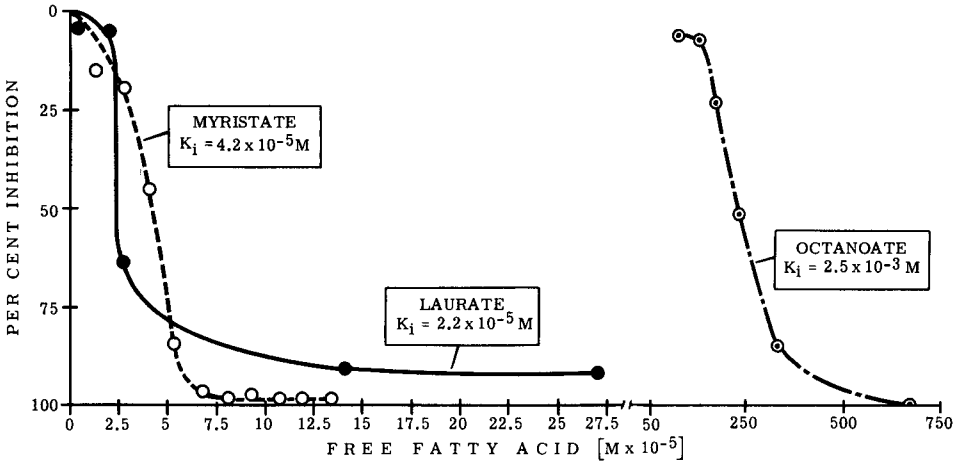

FIG. 2. Effect of increasing concentrations of FFA on liver pyruvate kinase. The final concentration of FFA in the assay reaction mixture is shown.

enzymes and a decrease or no change in the glycolytic ones. However, these changes in gluconeogenic conditions, characterized by a decrease in insulin level (i.e., starvation and diabetes) or after glucocorticoid injection, require a number of hours or days for full development of the pattern (13). The acute adaptation requires a rapid change in the enzyme ratios which cannot be accomplished by alterations in the rate of biosynthesis and degradation of the enzymes.

Such considerations directed our attention to the fact that under gluconeogenic conditions there is a rise in hepatic FFA and acetyl-CoA levels, and we investigated the possible mechanism of effect of these metabolites in carbohydrate metabolism. The investigation revealed that there is a network of metabolic signals, including FFA, acetyl-CoA, L-alanine, NADH, ATP, oxaloacetate, phosphoenolpyruvate and other metabolites, which are able to inhibit certain of the key enzymes of glucose catabolism and thus inhibit glycolysis and facilitate gluconeogenesis (8-12). Since FFA may be recognized as an end product of glucose breakdown and acetyl-CoA as the end product of FFA breakdown, the ability of FFA and acetyl-CoA to inhibit glucose catabolism may be called sequential feedback inhibition.

EXPERIMENTAL PROCEDURES

Male Wistar rats weighing 100-200 g were kept in separate cages; Purina laboratory chow and tap water were always available. The animals were killed by decapitation and exsanguination. The preparation of liver homogenate and supernatant fluid was carried out as described previously (14).

Most of the chemicals and auxilliary enzymes were purchased from Sigma Chemical Company. Lactate dehydrogenase was also purchased from Boehringer Company and acetyl-CoA from P & L Biochemicals, Inc. In most

TABLE I

Inhibition of Glucokinase and Hexokinase by Fatty Acids

Fatty acid	Concentration of fatty acid (M) for 50% inhibition of enzyme activity			
	Glucokinase		Hexokinase	
	Preincubation concentration	Assay concentration	Preincubation concentration	Assay concentration
Octanoate	5.0×10^{-3}	4.0×10^{-4}	3.6×10^{-2}	2.9×10^{-3}
Laurate	5.0×10^{-4}	4.0×10^{-5}	1.0×10^{-3}	8.0×10^{-5}
Myristate	1.4×10^{-4}	1.1×10^{-5}	2.4×10^{-3}	1.9×10^{-4}
Palmitate	9.4×10^{-4}	7.5×10^{-5}	a	a
Elaidate	3.0×10^{-4}	2.4×10^{-5}	a	a

[a]Not determined

TABLE II

Effect of Palmitate on Lactate Production

Preincubation time, min	Lactate production after preincubation in different media[a]		
	No palmitate	0.5 mM Palmitate	1.0 mM Palmitate
0	105 ± 15	92 ± 19	79 ± 16
5	104 ± 12	40 ± 9	22 ± 2
15	77 ± 13	27 ± 7	19 ± 3
30	68 ± 11	14 ± 4	8 ± 4

[a]Expressed in μmoles lactate produced/hr per gram tissue.

experiments the acetyl-CoA obtained from P & L was used, since this preparation is claimed to be 98% pure. The preparation of solutions and suspensions of FFA was described previously (10). The acetyl-CoA was dissolved in distilled water and adjusted to pH = 7.4, or it was dissolved in glycylglycine buffer, pH = 7.4. The assays of the liver enzymes and the method for determining lactate production were conducted as described previously (8-12).

The enzyme determinations were carried out under conditions giving zero order kinetics. It was carefully established that the auxilliary enzymes added to catalyze the coupled reactions did not become limiting in the enzyme assay. The FFA, acetyl-CoA, or phosphoenolpyruvate (PEP) was preincubated with the liver supernatant fluid at 37 C for 10 min or added directly to the assay reaction mixture, unless otherwise specified. Suitable controls were run at the same time and the assays were carried out in a Cary 11 recording spectrophotometer and in a Gilford Model 2000 spectrophotometer.

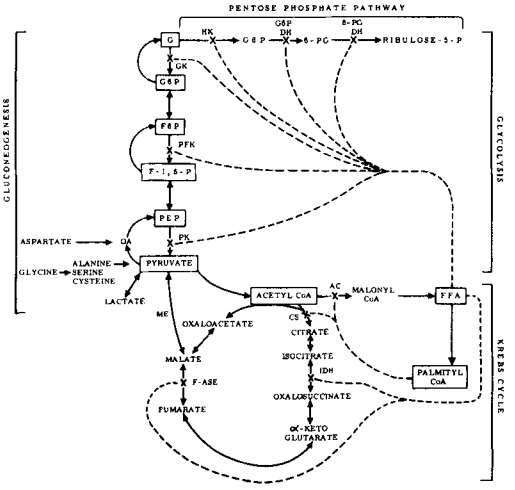

FIG. 3. Feedback inhibition by fatty acids of hepatic enzymes involved in glucose catabolism. GK, Glucokinase; PFK, Phosphofructokinase; PK, Pyruvate Kinase; HK, Hexokinase; G6P DH, Glucose-6-Phosphate Dehydrogenase; 6-PG DH, 6-Phosphogluconate Dehydrogenase; IDH, Isocitrate Dehydrogenase; F - Ase, Fumarase; ME, Malic Enzyme; AC, Acetyl CoA Carboxylase; and CS, Citrate Synthase.

RESULTS AND DISCUSSION

Effect of FFA on Key Glycolytic Enzymes and Lactate Production

The inhibitory effect of FFA on hepatic pyruvate kinase is illustrated in Figure 2. The short chain FFA, octanoate, gave a K_i = 2.5 X 10^{-3}M, whereas the physiologically more common, longer chain fatty acids were two magnitudes more effective in terms of final FFA concentration. The concentrations of FFA in preincubation mixture for octanoate, laurate and myristate were 3.7 X 10^{-2}, 3.3 X 10^{-4} and 6.3 X 10^{-4} molar respectively. These results confirm and extend our previous work on the inhibition of liver pyruvate kinase by FFA

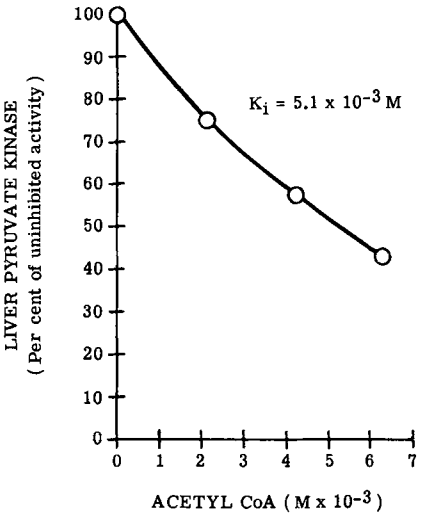

FIG. 4. Effect of addition of acetyl-CoA to assay reaction mixture of liver pyruvate kinase. The final concentrations of acetyl-CoA are shown.

(8,10). Further experimental details and considerations are provided in references 10, 12, 20 and 21.

The inhibition of glucokinase and hexokinase by fatty acids is reported in Table I. It may be seen that the long chain FFA are more effective than octanoate and they are all inhibitory to both enzymes of glucose phosphorylation. These results are in line with our previous studies which have also described the inhibition of hepatic phosphofructokinase by FFA (8-10).

Extensive studies demonstrated that octanoate did not affect the key gluconeogenic and bifunctional enzymes examined (8,10). The inhibitory action of FFA on key glycolytic enzymes implies that lactate production should be blocked. Our studies indicated that this is so (9,10) and Table II illustrates the effect of palmitate on lactate production. A 1.1 ml aliquot of 10% liver supernatant fraction was preincubated at 37 C with 1.3 ml of a solution containing 50 μmoles glycylglycine pH 7.4 and appropriate concentrations of palmitate to give concentrations of 0, 0.5 and 1.0 mM in the total volume of 2.4 ml. After stated preincubation times 0.4 aliquots from these mixtures were taken for determination of lactate production in the standard medium with a final volume of 1.0 ml. Each value is the mean and the standard error of the mean of assays on three animals. It may be seen that, depending on the preincubation time, a progressive inhibition of lactate production was registered. Previous work described the inhibition of lactate production by octanoate, myristate and laurate (9,10).

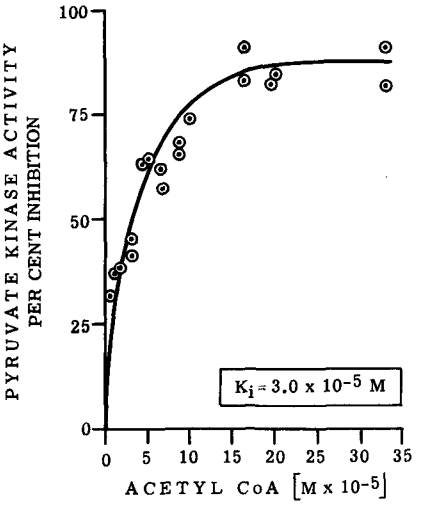

FIG. 5. Effect of acetyl-CoA on liver pyruvate kinase activity using a 10 min preincubation. In this dose response study, the final concentrations of acetyl-CoA are shown.

The Action of FFA as a Metabolic Switch

These results suggest a working hypothesis for the action of FFA in the regulation of carbohydrate metabolism. When in gluconeogenic conditions the FFA level is increased, this functions as a rapid inhibitor of the key glycolytic enzymes and as a stimulus for gluconeogenesis. The source of FFA from the periphery is chiefly the adipose tissue from which the lipolytic hormones, epinephrine, glucagon,

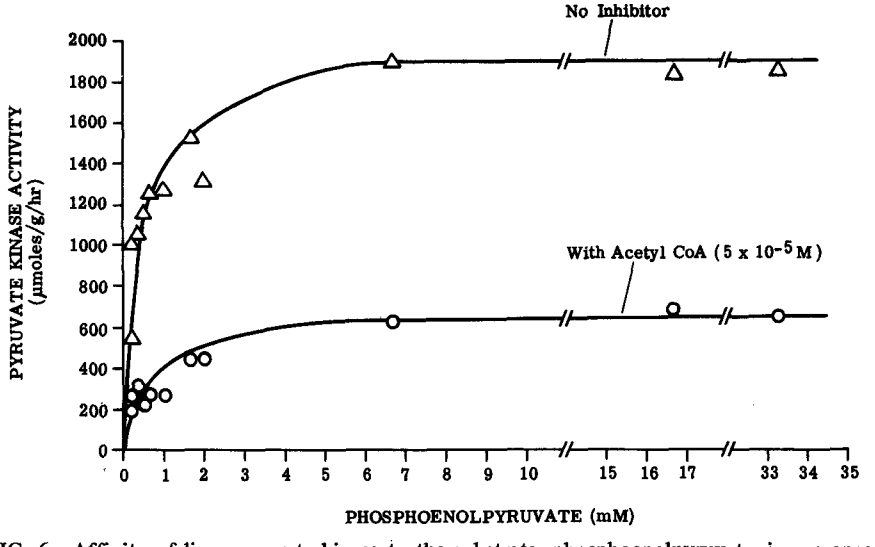

FIG. 6. Affinity of liver pyruvate kinase to the substrate, phosphoenolpyruvate, in presence and absence of acetyl-CoA (10-min preincubation was used).

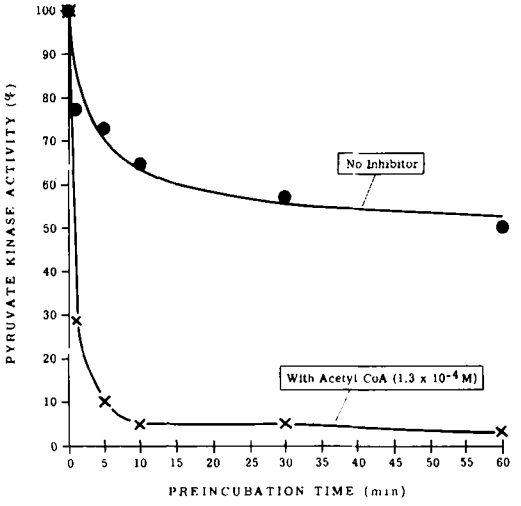

FIG. 7. Effect of preincubation time on hepatic pyruvate kinase activity in presence and absence of acetyl-CoA at 37 C.

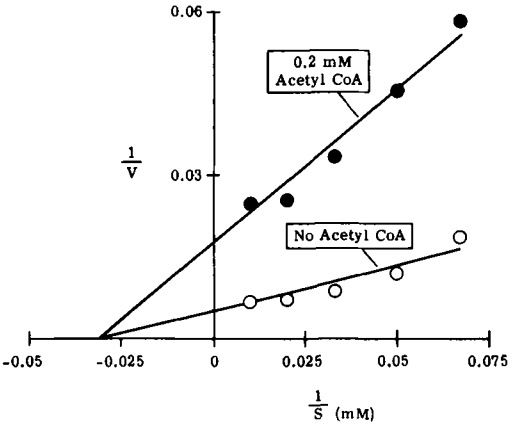

FIG. 8. Reciprocal plot showing the inhibition of liver glucokinase by acetyl-CoA.

ACTH, growth hormone and the glucocorticoids may cause a release of FFA into the plasma. The balance of these hormones with the anti-lipolytic hormone, insulin, is crucial in determining the FFA levels in liver and plasma.

The enzymatic findings and those obtained in the lactate-producing systems suggest an overall action for FFA in the regulation of carbohydrate metabolism which is illustrated in Figure 3. This Figure draws attention to the fact that FFA may be considered an end product in the metabolism of glucose. Our earlier observations (8,10) and the current work suggest that FFA may function as a feedback inhibitor by inhibiting the key enzymes of glycolysis and of the direct oxidative pathway (8-10,12). The selectivity of FFA action is also supported by our studies with enzymes of the Krebs cycle where octanoate inhibited isocitrate dehydrogenase and fumarase, but did not affect malate dehydrogenase and the malic enzyme. These results agree with reports on the increase in gluconeogenesis and on the decreased functioning of the Krebs cycle in the presence of a rise in the FFA concentration (1-7).

Effect of Acetyl-CoA on Key Glycolytic Enzymes

Since acetyl-CoA is the end product of FFA metabolism and its level increases under gluconeogenic conditions (15,16), it seemed possible that it may exert an inhibitory action on certain of the key glycolytic enzymes. A further indication for a role in metabolic control was also suggested by the fact that acetyl-CoA was a required cofactor in the activation of pyruvate carboxylase (17) which may be considered as the first step in the final common path of gluconeogenesis. Recent work showed that acetyl-CoA indeed was inhibitory to liver and muscle pyruvate kinase and it also inhibited hepatic glucokinase (11,12). The present work confirms and extends these studies. Figure 4 shows that when acetyl-CoA was added to the assay mixture it inhibited hepatic pyruvate kinase with a K_i = 5.1 X 10^{-3}M. When the liver supernatant fluid was preincubated with acetyl-CoA, this coenzyme was two magnitudes more effective as an inhibitor of liver pyruvate kinase, giving a K_i = 3.0 X 10^{-5}M (Figure 5). Concentrations above 0.15 mM resulted in an almost complete inhibition of this enzyme activity.

Mechanism of Inhibition of Liver Pyruvate Kinase by Acetyl-CoA. The nature of inhibition was studied using the preincubation system (10 min). Results indicated that the inhibition by acetyl-CoA did not cause a marked change in the affinity of hepatic pyruvate kinase to its substrate, phosphoenolpyruvate, and that it was not a competitive type of inhibition (Fig. 6).

Effect of Preincubation Time on Liver Pyruvate Kinase Activity Without and With Acetyl-CoA. It was of interest to determine the sequence of events when acetyl-CoA was added to the supernatant fluid, since preincubation was more effective for this inhibitory mechanism. Figure 7 shows that at 37 C the pyruvate kinase activity decreased to near 50% in 60 min. In contrast, when acetyl-CoA (1.3 X 10^{-4}M) was included in the preincubation mixture, the enzyme activity decreased to 30% in 1 min and by 10 min it was barely measurable. Thus, the inhibitory action of acetyl-CoA requires very little time for the interaction. This mechanism is under further investigation.

TABLE III

Inhibition of Glucokinase Activity by Acetyl Coenzyme A

Acetyl coenzyme A in assay (mM)	Glycose phosphorylated μmoles	Per cent inhibition
0.00	104	0
0.02	84	19
0.04	76	27
0.08	49	53
0.12	43	59
0.16	35	66
0.20	26	75

Inhibition of Glucokinase Activity by Acetyl-CoA. Table III shows that increasing concentrations of acetyl-CoA in the 10 min preincubation mixture caused a progressive inhibition of liver glucokinase activity, yielding a $K_i = 7.5 \times 10^{-5}$M (11). A 0.25 ml aliquot of 10% liver supernatant fraction was preincubated for 10 min at 37 C with 0.25 ml 0.5 M glycylglycine pH 7.4 and appropriate concentrations of acetyl coenzyme A. From this mixture, 0.1 ml aliquots were taken for assay of glucokinase activity in a final volume of 2.5 ml.

Mechanism of the Inhibition of Glucokinase by Acetyl-CoA. We explored the mechanism of inhibition by using the preincubation system, and investigated the affinity of liver glucokinase to the substrate, glucose, in the absence and in the presence of acetyl-CoA (0.2 mM). Such studies are illustrated in Figure 8 which shows that the inhibition of hepatic glucokinase by acetyl-CoA is affected through a noncompetitive mechanism.

Selectivity of the Inhibitory Action of Acetyl-CoA. As in the case of the FFA, in order to assume a physiological role for the inhibitory action of acetyl-CoA it is necessary to establish a selectivity of action. Our investigations showed that in the concentration range where acetyl-CoA was inhibitory to liver glucokinase and pyruvate kinase no inhibition was observed against liver hexokinase, phosphofructokinase, lactate dehydrogenase, glucose-6-phosphatase and fructose 1,6-diphosphatase (11,12). Thus, two key gluconeogenic enzymes and lactate dehydrogenase, an enzyme which plays a part in gluconeogenesis, are not affected, which is in accord with the role we postulate for acetyl-CoA in inhibiting glycolysis and stimulating gluconeogenesis. The fact that liver phosphofructokinase was not inhibited by acetyl-CoA indicates a higher selectivity for the action of acetyl-CoA than for that observed for FFA because the latter also inhibited hexokinase and phosphofructokinase in the same concentration range in which it was inhibitory for glucokinase and pyruvate kinase (11,12). The absence of an inhibition of liver hexokinase indicates that the auxilliary enzyme used in this assay and in the glucokinase assay, namely yeast glucose-6-phosphate dehydrogenase, was also not inhibited by acetyl-CoA. Similarly, the auxilliary enzyme used in the pyruvate kinase assay, namely, rabbit muscle lactate dehydrogenase, was also not subject to inhibition by acetyl-CoA (11,12).

Possible Physiological Significance of the Inhibitory Effects of Acetyl-CoA. Since acetyl-CoA activates pyruvate carboxylase (17), a simultaneous inhibition of the antagonistic enzyme, pyruvate kinase, provides a rapid mechanism by which metabolic channeling can be accomplished with economy and speed (11,12). As a result acetyl-CoA may be able to provide at least three regulatory functions at the enzyme activity level. These are: activation of a key enzyme of gluconeogenesis, pyruvate

TABLE IV

Inhibition of Glucokinase by Phosphoenolpyruvate

Phosphoenolpyruvate in assay (mM)	Glucose phosphorylated (μmoles/hr/gram tissue	Per cent inhibition
0.0	132	0
0.8	105	20
1.6	86	35
2.4	51	61
3.2	29	78
4.0	16	88
4.8	8	94

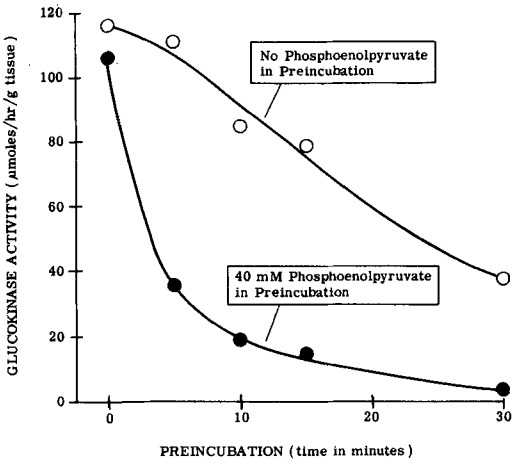

FIG. 9. Effect of preincubation time on hepatic glucokinase activity in presence and absence of phosphoenolpyruvate at 37 C.

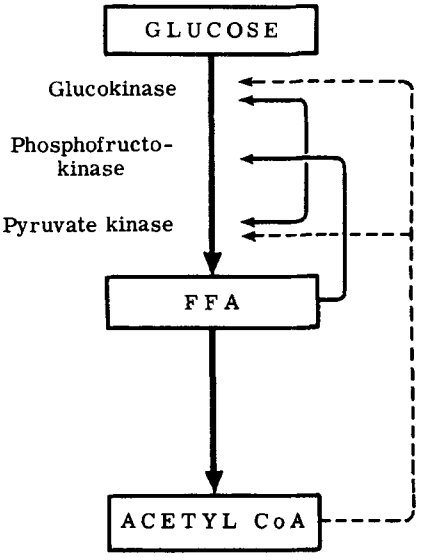

FIG. 10. Sequential inhibition of hepatic key glycolytic enzymes by FFA and acetyl-CoA.

carboxylase: prevention of the recycling of PEP by inhibiting a glycolytic enzyme, pyruvate kinase; and blocking the utilization of pyruvate by inhibition of the enzyme, pyruvate oxidase. The concentration range of hepatic acetyl-CoA for these effects appears to be in the physiological order of magnitude which for acetyl-CoA is 20-60 μM (15,16); the affinity of pyruvate carboxylase to acetyl-CoA is K_m = 19 μM (17) and for the inhibitory action of acetyl-CoA on pyruvate kinase the K_i = 30 μM.

Acetyl-CoA is the end product of the degradation of FFA which in turn arises as an end product of glucose catabolism; therefore, the inhibition of the three key glycolytic enzymes by FFA and the subsequent reinforcement of the inhibition of glucokinase and pyruvate kinase by acetyl-CoA may be called sequential feedback inhibition.

Phosphoenolpyruvate: Inhibitor of Glucokinase

In an investigation of the possible effects of positive and negative signals by metabolites, the action of PEP on glucokinase was examined. When the supernatant fraction of liver was preincubated for 10 min with increasing concentrations of PEP, a progressive inhibition of glucokinase activity was observed (Table IV). Under these conditions an almost complete inhibition was observed with a PEP concentration of 4.8 mM and a K_i = 2.1 mM was found (11,12). A 0.5 ml aliquot of 10% rat liver supernatant fraction was preincubated at 37 C for 10 min with 1.0 ml of a solution containing 50 μmoles glycylglycine pH 7.4 and different concentrations of phosphoenolpyruvate. From this mixture 0.2 ml aliquots were taken for assay of glucokinase activity in a final volume of 2.5 ml.

Mechanism of Action of PEP on Hepatic Glucokinase. The affinity of glucokinase to its substrate, glucose, was examined in presence and absence of PEP. Using a preincubation system of 10 min the concentration of PEP was 1.07 mM. When PEP was added directly to the enzyme assay mixture, it was given in a concentration of 20 mM. The results indicated that the affinity of glucokinase to glucose (K_m = 2.8 X 10^{-2}M) was the same in the presence and in the absence of PEP. These experiments demonstrated that the inhibition of liver glucokinase by PEP was of the noncompetitive type (11,12).

Effect of Preincubation Time With Phosphoenolpyruvate on Hepatic Glucokinase Activity. It was of interest to determine the sequence of events when PEP was added to the supernatant fluid, as preincubation was more effective for the inhibitory mechanism. Figure 9 shows that when the liver supernatant fluid was preincubated with distilled water (pH = 7.4) at 37 C the glucokinase activity decreased to approximately 40% in 30 min. However, when PEP (40 mM) was included in the preincubation mixture, the glucokinase activity declined to less than 40% in 5 min. It appears that the inhibitory action of PEP requires only a few minutes, and that the effect is a progressive one with incubation time.

Possible Physiological Significance of the Inhibition of Glucokinase by PEP. The effective concentration of PEP for the inhibition of these

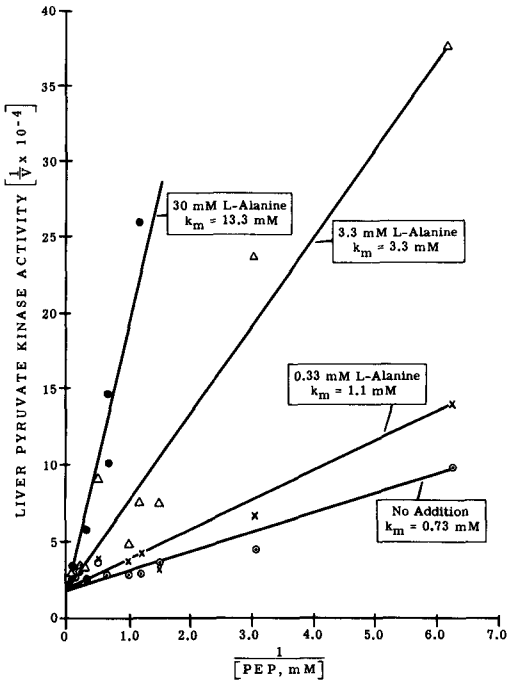

FIG. 11. Affinity of liver pyruvate kinase to its substrate, phosphoenolpyruvate, in presence and absence of different concentrations of L-alanine.

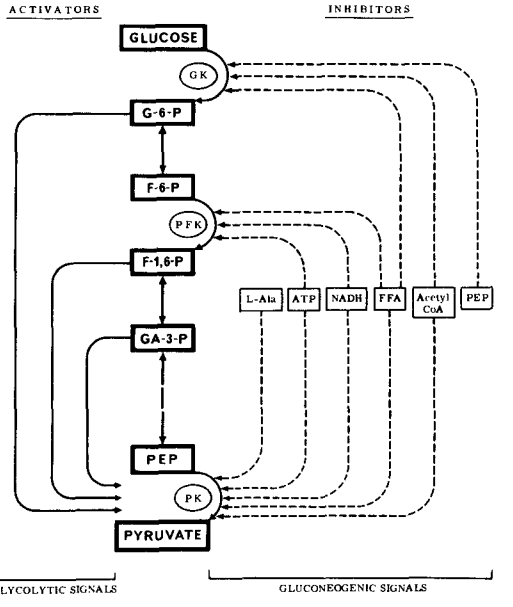

FIG. 12. Regulation of pyruvate kinase and the other key glycolytic enzymes, glucokinase and phosphofructokinase. The array of activators controlling pyruvate kinase and the inhibitors regulating the various key glycolytic enzymes are shown.

enzymes is several magnitudes higher than the level of PEP in the liver. It is likely that for the action of PEP to be of physiological significance it must work in a cooperative manner with other inhibitors of glucokinase, such as FFA and acetyl-CoA, the levels of which increase in gluconeogenic conditions. Then PEP may act on a partially inhibited enzyme and a much lower PEP concentration may be effective. It is interesting that there is a selectivity in the effects of PEP because liver hexokinase is not affected.

Current View on the Postulated Roles of Lipid Metabolites and Other Metabolites on Regulation of the Direction of Carbohydrate Metabolism in Liver

The results presented, along with the previous data obtained in our laboratories, provide an explanation, in part at least, for the stimulatory effect of FFA and acetyl-CoA on hepatic gluconeogenesis. Under gluconeogenic conditions, the resulting rise in FFA and the subsequent one in acetyl-CoA should inhibit the key glycolytic enzymes. Since FFA is an end product of glucose catabolism, and in turn acetyl-CoA is an end product of FFA degradation, we termed this regulatory mechanism sequential feedback inhibition (Fig. 10).

The inhibition of pyruvate kinase is of physiological significance because this glycolytic enzyme is many times more active than the opposing gluconeogenic enzyme, pyruvate carboxylase (Fig. 1). Therefore, it is relevant that several inhibitors of liver pyruvate kinase activity have been observed (Fig. 12). The amount of liver pyruvate kinase will decrease with the decline of insulin level on which the biosynthesis of this enzyme appears to depend, in part at least (8,18). The activity of liver pyruvate kinase is inhibited by NADH (10,19), ATP (10), L-alanine (12) (see Fig. 11), FFA (8-10) and acetyl-CoA (11,12). Thus, there is an array of inhibitors which may provide cooperative or cumulative inhibition on pyruvate kinase and on other key enzymes of glycolysis, glucokinase and phosphofructokinase, depending on the nature and extent of the gluconeogenic conditions (see Fig. 12). Once phosphoenolpyruvate is formed, it should signal the process of gluconeogenesis and should decrease phosphorylation by inhibiting the activity of glucokinase, which enzyme has already been informed by FFA and acetyl-CoA that gluconeogenesis was in process (20,21). Thus, the gluconeogenic pathway will predominate over glycolysis in the liver.

ACKNOWLEDGMENTS

This investigation was supported by grants from the United States Public Health Service, the National Cancer Institute (CA-05034), the American Cancer Society and the Damon Runyon Memorial Fund.

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[Received September 27, 1968]

SHORT COMMUNICATIONS

Human α-2 Lipoprotein Bands Visualized by Cellulose Acetate Electrophoresis

Separation of human serum lipoproteins by cellulose acetate electrophoresis has been previously described (1,2). During investigations of human lipoprotein metabolism, the cellulose acetate electrophoretic pattern of certain subjects was found to contain a double band in the α_2 region, as shown in Figure 1. This consisted of a component comparable to the pre-β lipoproteins of paper electrophoresis (1-3) and a slower moving α_1 component not previously described.

Plasma lipoproteins were separated on cellulose acetate using barbital buffer pH 8.6, ionic strength 0.075 with 1% albumin and 0.001 M EDTA. Five microliters of plasma from fasting subjects, some with known hyperlipoproteinemias, was applied to cellulose acetate strips (Gelman Sepraphore III, 6 ft x 1 in.; Gelman Inst. Co., Ann Arbor, Mich.), pre-soaked in buffer at 4 C. Electrophoresis was run at 250 v for 90 min at room temperature and stained in Oil Red O with 70% methanol at 37 C for 8-12 hr. They were rinsed in a 5% solution of acetic acid, photographed while wet with a Poloroid Land Camera MP-3 and then cleared in 15% acetic acid-methanol solution.

Individual fractions for electrophoresis were prepared in the Beckman Model L ultracentrifuge (4). The fractions separated were: $\sigma <$ 1.006 g/ml (chylomicrons), $\sigma <$ 1.006 g/ml (pre-β lipoproteins), $\sigma >$ 1.006 g/ml & $< \sigma$ 1.063 /ml. (β-lipoproteins) and $> \sigma$ 1.063 g/ml. (α-lipoproteins). These individual fractions were subjected to cellulose acetate electrophoresis. In addition, α-lipoproteins were prepared by adding 0.15 ml 1 M manganese chloride and 6 mg of sodium heparin to 3 ml of plasma. The supernatant was removed after centrifugation at 2000 rpm for 15 min at 4 C. This supernatant contains lipoproteins immuno-chemically identical to α-lipoproteins (5). Plasma specimens were also studied after they had been stored at 4 C for various lengths of time or had been subjected to several ultracentrifugations.

All subjects showing a slower moving α_1 band had moderately elevated plasma cholesterol (range 280-350 mg/100g and normal to moderately elevated triglycerides (75-380 mg/100g. The slower migrating α_1 component was not seen in 100 subjects with plasma cholesterols < 250 mg/100g and was rarely seen in subjects with carbohydrate inducible hyperlipoproteinemia (type IV Fredrickson classification).

The slower moving component of the α bands was isolated in the preparative ultracen-

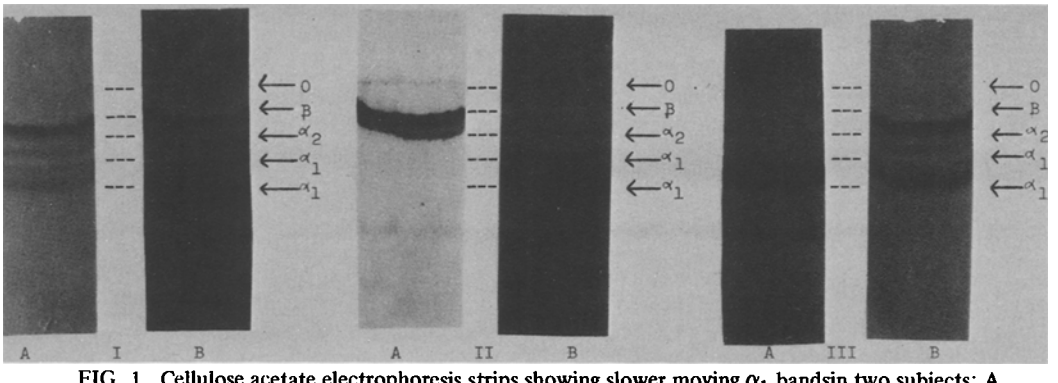

FIG. 1. Cellulose acetate electrophoresis strips showing slower moving α_1 bandsin two subjects: A. Cholesterol, 258 mg/100 g; Triglycerides, 126 mg/100 g; B. Cholesterol, 348 mg/100 g, Triglycerides: 103 mg/100 g. II. Electrophoresis strips showing separation of the α_2 and slower moving α_1 band by preparative ultracentrifugation: A. $\sigma < 1.063$; B. $\sigma > 1.063$/ml. III. Electrophoresis of the supernatant after the low density lipoprotein removal by precipitation with manganese chloride-heparin solution: A. Supernatant showing α_1 bands; B. Control showing α_2 and slower α_1 component.

trifuge at $\sigma > 1.063$ g/ml while the pre-β component was isolated at $\sigma < 1.006$ g/ml as expected (Fig. 1). When low density lipoproteins were precipitated with 1 M manganese chloride and sodium heparin, the slower moving component of the α bands remained in the supernatant as shown in Figure 1. Storing plasma at 4 C did not noticeably increase the intensity of the fast components nor did repeated ultracentrifugation.

These preliminary studies show that the slower moving component of the α band has different physical characteristics from the α_2 band as determined by these two techniques. The α_2 component is identified as the pre-β lipoproteins of paper electrophoresis while slower α_1 has some of the characteristics of α or high density lipoproteins. These differences could be due to different lipid-peptide ratios or to the presence of different peptides in each component.

Studies are under way to further delineate the characteristics of this faster band and its relationship to other lipoproteins in the high and very low density spectrum.

R. C. CHARMAN
C. L. BRAEULER
Department of Medicine, Hitchcock Clinic
Hanover, New Hampshire 03755

ACKNOWLEDGMENT

This work was supported in part by a grant from the Hitchcock Foundation.

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[Received January 27, 1969]

Methyl Octadecadienoates as Protectors Against Inactivation of Alkaline Phosphatase by γ-Radiation

ABSTRACT

Methyl *cis,cis-*, *cis,trans-* and *trans,trans*-9,12-octadecadienoates protect alkaline phosphatase from inactivation by γ-rays. The three isomers are equally efficient.

Polyunsaturated fatty acids or their derivatives are quite sensitive to high energy radiations (1) and react readily with many free radicals. Therefore, it might be anticipated that these lipids could modify the effects of ionizing radiations on other sensitive compounds. We used alkaline phosphatase to test this and found that methyl linoleate and its *trans* isomers protect it from damage by γ-rays.

A solution of alkaline phosphatase was prepared by dissolving 1 mg of dry enzyme preparation (Type II, Sigma Chemical Co., St. Louis) in 50 ml of 0.02 phosphate buffer of pH 7.5. Five to ten milliliters of the enzyme solution were emulsified with 1% to 5% of methyl *cis,cis-*, *cis,trans-* or *trans,trans*-9,12-octadecadienoate, using 5% propylene glycol to stabilize the emulsions. Stable emulsions were obtained by blending the components for 3 min in an Omni Mixer in an atmosphere of nitrogen. Controls without methyl esters, but containing 5% propylene glycol, were prepared in the same manner.

The samples were irradiated within 20 hr of their preparation. They were kept at 3 C except during irradiation which was performed at 22 C with 50 or 500 kilorads of γ-rays from a ^{137}Cs source at a dose rate of 412 kilorads/hr. Non-irradiated duplicates of all samples were kept at room temperature while the samples were irradiated, but were otherwise treated identically and were analyzed in parallel with the irradiated specimens. Alkaline phosphatase was determined by the spectrophotometric method of Bessey et al. (2) as standardized by Sigma Chemical Co. (1963). The samples containing lipids were not optically transparent and the fat was removed prior to analysis by a single extraction with petroleum ether. To assure uniformity, all samples, whether they contained added lipids or not, were subjected to this extraction.

In preliminary experiments it was established that enzyme solutions stored for eight days at 3 C lost 19% of their original activity. Emulsification of the enzyme solution and

trifuge at $\sigma > 1.063$ g/ml while the pre-β component was isolated at $\sigma < 1.006$ g/ml as expected (Fig. 1). When low density lipoproteins were precipitated with 1 M manganese chloride and sodium heparin, the slower moving component of the α bands remained in the supernatant as shown in Figure 1. Storing plasma at 4 C did not noticeably increase the intensity of the fast components nor did repeated ultracentrifugation.

These preliminary studies show that the slower moving component of the α band has different physical characteristics from the α_2 band as determined by these two techniques. The α_2 component is identified as the pre-β lipoproteins of paper electrophoresis while slower α_1 has some of the characteristics of α or high density lipoproteins. These differences could be due to different lipid-peptide ratios or to the presence of different peptides in each component.

Studies are under way to further delineate the characteristics of this faster band and its relationship to other lipoproteins in the high and very low density spectrum.

R. C. CHARMAN
C. L. BRAEULER
Department of Medicine, Hitchcock Clinic
Hanover, New Hampshire 03755

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Polyunsaturated fatty acids or their derivatives are quite sensitive to high energy radiations (1) and react readily with many free radicals. Therefore, it might be anticipated that these lipids could modify the effects of ionizing radiations on other sensitive compounds. We used alkaline phosphatase to test this and found that methyl linoleate and its *trans* isomers protect it from damage by γ-rays.

A solution of alkaline phosphatase was prepared by dissolving 1 mg of dry enzyme preparation (Type II, Sigma Chemical Co., St. Louis) in 50 ml of 0.02 phosphate buffer of pH 7.5. Five to ten milliliters of the enzyme solution were emulsified with 1% to 5% of methyl *cis,cis-*, *cis,trans-* or *trans,trans*-9,12-octadecadienoate, using 5% propylene glycol to stabilize the emulsions. Stable emulsions were obtained by blending the components for 3 min in an Omni Mixer in an atmosphere of nitrogen. Controls without methyl esters, but containing 5% propylene glycol, were prepared in the same manner.

The samples were irradiated within 20 hr of their preparation. They were kept at 3 C except during irradiation which was performed at 22 C with 50 or 500 kilorads of γ-rays from a ^{137}Cs source at a dose rate of 412 kilorads/hr. Non-irradiated duplicates of all samples were kept at room temperature while the samples were irradiated, but were otherwise treated identically and were analyzed in parallel with the irradiated specimens. Alkaline phosphatase was determined by the spectrophotometric method of Bessey et al. (2) as standardized by Sigma Chemical Co. (1963). The samples containing lipids were not optically transparent and the fat was removed prior to analysis by a single extraction with petroleum ether. To assure uniformity, all samples, whether they contained added lipids or not, were subjected to this extraction.

In preliminary experiments it was established that enzyme solutions stored for eight days at 3 C lost 19% of their original activity. Emulsification of the enzyme solution and

TABLE I

Activity of Irradiated Alkaline Phosphatase Solutions

Dose	Storage	Methyl ester added			No methyl ester added
Kilorads	Days	Methyl ester	Conc.	Activity,%[a]	Activity[a]
Experiment 1					
50	2	*cis,cis*	2	76	
50	2	*cis,trans*	2	76	8
50	2	*trans,trans*	2	76	
50	8	*cis,cis*	2	83	
50	8	*cis,trans*	2	84	2
50	8	*trans,trans*	2	59	
500	2	*cis,cis*	2	51	
500	2	*cis,trans*	2	59	1
500	2	*trans,trans*	2	45	
500	8	*cis,cis*	2	44	
500	8	*cis,trans*	2	39	0
500	8	*trans,trans*	2	27	
Experiment 2					
50	2	*cis,cis*	1	43	
50	2	*cis,cis*	3	28	
50	2	*cis,cis*	5	86	4
50	2	*cis,trans*	5	87	
50	2	*trans,trans*	5	79	
50	8	*cis,cis*	1	11	
50	8	*cis,cis*	3	39	
50	8	*cis,cis*	5	79	0
50	8	*cis,trans*	5	58	
50	8	*trans,trans*	5	70	
Experiment 3					
50	2	*cis,cis*	1	43	
50	2	*cis,cis*	3	48	
50	2	*cis,cis*	5	52	47
50	2	*cis,trans*	5	80	
50	2	*trans,trans*	5	125	
50	8	*cis,cis*	1	37	
50	8	*cis,cis*	3	6	
50	8	*cis,cis*	5	42	7
50	8	*cis,trans*	5	44	
50	8	*trans,trans*	5	39	
			Mean	57.12	8.62
			S. D.	25.63	15.80
			P	< 0.001	

[a]Expressed as per cent of activity of corresponding nonirradiated sample.

extraction of the lipids with petroleum ether resulted in a 16% loss of activity.

The data in Table I were obtained from three sets of experiments performed several weeks apart. The initial activities of the enzyme solutions used for each of these experiments were 2.12, 1.46 and 4.63 Sigma units/ml. Because of these variations resulting from factors other than irradiation, the activities of the irradiated samples in Table I have been expressed as per cent of the corresponding nonirradiated but otherwise identical samples.

The table shows the enzyme activity remaining, after irradiation, in 32 samples containing methyl esters and in eight controls to which no lipids were added. When the values for these two groups are pooled separately regardless of dose, post-irradiation storage time and kind and concentration of added methyl ester, the average activity of the samples containing added lipids (57.12) is very significantly different ($P < 0.001$) from the activity of the samples containing no ester (8.62). The protection factor, a quantitative measure of protection due to lipids, is given by the ratio of the activity of an irradiated sample containing lipid

to the activity of the corresponding irradiated sample without lipids. The overall average protection factor determined on this basis is 5.56 (57.12/8.62).

Increasing the dose 10-fold to 500 kilorads or the post-irradiation storage period to eight days resulted in significant further decreases in enzyme activity. In both cases the protection factors were increased, indicating that the lipids were more effective protectors under the more drastic conditions.

In two sets of experiments 1%, 3% and 5% *cis,cis*-linoleate were employed to study the effect of lipid concentration on enzyme protection. Although in all cases highest protection is associated with the 5% concentration, the differences between the three concentrations were not statistically significant. Also, there was no difference between the three isomers studied.

The protection mechanism is not known. The polyunsaturated methyl esters may react with the highly oxidizing HO· and HOO· free radicals which are the major primary products from irradiation of aqueous solutions. The products may be nonradicals or less reactive free radicals incapable of oxidizing the tyrosine residue believed to be associated with the activity of alkaline phosphatase (3).

TOSHIMI AKIYA[1]
T. H. MADSON
J. R. CHIPAULT
University of Minnesota
The Hormel Institute
Austin, Minnesota 55912

[1]Research Fellow at The Hormel Institute 1966-1967. Present address: Food Research Institute, Ministry of Agriculture and Forestry, 1-4-12 Shiohamacho, Koto-ku, Tokyo, Japan.

ACKNOWLEDGMENTS

This research was supported by The Hormel Foundation, U.S. Public Health Service Grant HE 08214, Program Projects Branch, National Heart Institute, and by Research Grant RH 00358, National Center for Radiological Health. John Sweet performed the irradiations.

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[Received May 20, 1969]

Studies on the Role of Phospholipids in the Triglyceride cycle: I. Phospholipid Synthesis in Liver and Plasma of Ethionine-Treated Rats[1]

C. A. OLMSTED, Department of Biological Sciences, Louisiana State University in New Orleans, Lake Front, New Orleans, Louisiana 70122

ABSTRACT

A single administration of *dl*-ethionine to rats was found to initiate a rapid accumulation of triglyceride in the liver while plasma and liver phospholipid (PL) levels decreased. As measured by the incorporation of intravenous $NaH_2P^{32}O_4$ at several time intervals after a single administration of ethionine, the drug decreased the specific activity (SA) of liver and plasma phospholipid phosphorus (PLP). At early time intervals after the injection of ethionine this occurred to a greater extent in the plasma than in the liver. Hence, the relative specific activity (RSA) of plasma to liver PLP was decreased initially by ethionine. As a measure of the exchange of PL between plasma and liver, this change in the RSA indicates decreased mobilization of PL from the liver to the plasma following ethionine inhibition of PL synthesis by the liver. Subsequently, the RSA increased, that is, an increased mobilization of PL occurred under conditions of fasting several hours after ethionine administration. Thus, it appeared that remobilization of liver PL tends to occur following the hepatic triglyceride accumulation brought about by inhibition of PL synthesis in the liver. However, remobilization of liver PL occurred also in male rats given ethionine and in fasted rats which did not show accumulation of hepatic triglyceride. These data suggest that phospholipid turnover and transport play an active role in the triglyceride cycle which is activated by fasting and during the recovery phase following ethionine administration.

INTRODUCTION

A continual mobilization of nonesterified fatty acids (NEFA) from adipose tissue to the liver has been demonstrated (1,2) and appears to provide substrate for oxidation and for resynthesis of fatty acids into triglycerides (TG) and other lipid moieties (3). The NEFA are synthesized into liver TG which are transported to the blood in the form of lipoproteins; this, plus the subsequent uptake of lipoproteins into adipose tissue and other organs has been designated as the triglyceride cycle (4-6). The composition of lipoprotein molecules varies with a number of physiological and pathological conditions (7) but all contain TG, phospholipids (PL), NEFA, cholesterol and sterol esters in addition to the protein portion of the complex.

Because plasma PL is an important constituent of lipoproteins, a study of the synthesis and exchange of liver and plasma PL seemed to be of importance in relation to nutritional and other factors which alter liver lipid mobilization. The uptake of inorganic radiophosphate ($NaH_2P^{32}O_4$) into liver and plasma PL was measured in male and female rats following a single administration of ethionine. The per cent incorporation and the specific activity (SA) of plasma and liver PL was measured and the ratio of the SA of plasma PL phosphorus (PLP) to liver PLP, designated as the relative specific activity (RSA), was used as a measure of liver PL mobilization.

EXPERIMENTAL PROCEDURES

Animal Care and Ethionine and Radiophosphate Dosages

Animals. Male and female Sprague-Dawley rats, fed Purina Laboratory Chow and given water ad libitum, were used throughout this investigation. The rats were 3 to 6 months of age at the time of use for each experiment, and were selected and paired on the basis of weight. Fed rats, and rats which had previously been fasted for a specified period of time, were used to control nutritional status. Initial body weight of control and experimental groups did not vary more than 10% in each of five experiments.

Ethionine Dosage. To obtain maximal effects, and to establish a more quantitative relation between dose and the time of biochemical changes in livers and plasma of animals used in this study, the *dl*-ethionine was administered by giving a single, intraperitoneal injection. The ethionine dosage, 1 g/kg body weight, was administered in a volume ranging

[1]Presented at the 58th Annual AOCS Meeting, New Orleans, May 1967.

TABLE I

Effect of *dl*-Ethionine on Total Liver Lipids and Total Phospholipids of Liver and Plasma of Fasted Female Rats

Exp. no.	Time fasted	Time after ethionine	No. of rats	Total liver lipids	Phospholipids	
					Liver	Plasma
	(hr)	(hr)		g % ± SD	mg-P/g ± SD	mg % ± SD
I	16	0	4	6.66 ± 0.47	1.43 ± 0.02	8.06 ± 0.30
	20	0	4	6.53 ± 0.71	1.38 ± 0.03	7.62 ± 0.30
	24	0	4	5.96 ± 0.37	1.28 ± 0.06	6.75 ± 0.90
	16	4.5	4	8.60 ± 0.85	1.45 ± 0.04	7.37 ± 0.60
	20	9	4	11.74 ± 1.64	1.17 ± 0.12	5.00 ± 0.70
	24	12	4	11.28 ± 1.06	1.14 ± 0.14	4.87 ± 1.40
II	24	0	6	5.12 ± 0.32	1.37 ± 0.09	3.10 ± 0.50
	27	3	2	5.10 ± 0.54	1.26 ± 0.09	2.87 ± 0.67
	24	5	2	6.62 ± 0.31	1.33 ± 0.05	1.90 ± 0
	30	6	2	8.41 ± 0.46	1.17 ± 0.11	2.37 ± 0

from 7-10 ml of 2.5% (w/v) solution of *dl*-ethionine in 0.154 M NaCl at 35 C. Although this concentration is a saturated solution, the ethionine solutions were prepared by weight rather than by saturation. Control rats received a single intraperitoneal injection of normal saline equal in volume to the volume of saturated *dl*-ethionine given to corresponding experimental groups.

The dosage used corresponds to that used in other studies on ethionine in rats except that here it was administered as a single dose rather than divided over a period of time as done by Simpson et al. (14).

Radiophosphate Administration. Inorganic radioactive phosphorus was injected intravenously as a high specific activity solution of $NaH_2P^{32}O_4$ in 1.0 ml of 0.154 M NaCl containing 100 μC of the radiophosphate.

Experimental Design. In the first series of experiments, measurements were made on total liver lipids and on total phospholipids of liver and plasma in 36 female rats under conditions of fasting and ethionine administration. In the second series of experiments, the 3-hr uptake of radiophosphate into liver and plasma phospholipids was measured in addition to the total liver lipids and total phospholipids of liver and plasma in 22 female rats and 16 male rats. In each experiment the animals were killed at several time intervals after ethionine administration, and after fasting in corresponding control groups.

Chemical Analyses

Plasma Phospholipids. Plasma phospholipids were extracted using alcohol and ether as previously described (8). The resultant precipitation of plasma proteins provided by the alcohol-ether extraction procedure was found to effect a coprecipitation of inorganic phosphorus; less than 1 X 10^{-6} % of inorganic radiophosphate added to plasma was found in the phospholipid fraction after using this method. Aliquots of the filtrate were dried and wet ashed with 70% perchloric acid[1] for the determination of the phospholipid phosphorus (PLP^{31}) by the method of King (9) and other aliquots were removed for radiochemical analyses.

Liver Lipids. Two gram portions of liver were homogenized prior to lipid extraction using alcohol and ether. The extracts were filtered through Whatman No. 1 filter paper and aliquots were taken for gravimetric determination of total lipids, for PLP^{31} determination by the method of King (9), and for radiochemical analyses.

It appears that the technique used for homogenization divided the liver tissue so finely that liver protein precipitation in the alcohol-ether coprecipitated inorganic phosphorus in much the same way as was observed in the case of plasma. Dissolving the liver lipid extracts in petroleum ether after drying under nitrogen and washing of the ether solution with weak aqueous solutions of nonradioactive sodium phosphate and with water did not change the specific activity of the phospholipid phosphorus in either the liver or the plasma lipid extracts.

Radiochemical Analyses

Per cent Injected Dose (%ID). The activity in each sample was referred to the injected dose (ID) as a standard, and the cpm of each fraction

TABLE II

Effect of *dl*-Ethionine on Total Liver Lipids and on Total Phospholipids of Liver and Plasma of Male and Female Rats

Expt. no.	Time fasted	Time after ethionine	Sex	No. of rats	Total liver lipids	Phospholipids	
						Liver	Plasma
	(hr)	(hr)			g % ± SD	mg-P/g ± SD	mg % ± SD
III	24	0	F	2	4.96 ± 0.41	1.32 ± .14	3.31 ± .11
	48	0	F	2	6.44 ± 0.13	1.20 ± .13	4.20 ± .17
	24	6	F	2	7.91 ± 0.46	1.17 ± .11	2.31 ± .11
	48	24	F	2	13.47 ± 0.05	0.93 ± .03	1.81 ± .12
	48	24	M	2	6.44 ± 1.35	1.20 ± .04	1.43 ± .22
IV	3	0	F	3	6.10 ± 0.03	1.15 ± .05	6.65 ± .68
	6	0	F	2	5.50 ± 0.09	1.06 ± .08	5.87 ± .03
	24	0	F	2	5.50 ± 0.44	1.05 ± .03	6.15 ± 0
	3	3	F	3	6.30 ± 0.05	1.16 ± .06	5.07 ± .06
	6	6	F	2	8.50 ± 0.44	1.12 ± .02	4.58 ± .15
	24	24	F	2	18.60 ± 2.40	0.85 ± .07	1.72 ± .21
V	3	0	M	3	5.70 ± 0.35	1.15 ± .13	5.86 ± 0.84
	6	0	M	2	5.10 ± 1.24	1.09 ± .11	6.37 ± 1.11
	24	0	M	2	5.00 ± 0.44	1.15 ± .06	3.81 ± 0.02
	3	3	M	3	5.50 ± 0.30	1.02 ± .05	6.34 ± 1.18
	6	6	M	2	5.20 ± 0.09	1.19 ± .03	5.30 ± 1.06
	24	24	M	2	6.80 ± 0.71	1.15 ± .11	3.24 ± 0.49

expressed as a per cent of the ID either per gram of liver, or per milliliter of plasma.

Specific Activity (SA). The SA of PLP was calculated as the ratio of PLP^{32} cpm per mg PLP^{31} and was expressed as a per cent of the ID. Calculation of this data as a %ID was done to enable the subsequent calculation of relative specific activity. Thus,

$$SA = PLP^{32} \text{ cpm/mg } PLP^{31}/ID \times 100$$

Relative Specific Activity (RSA). RSA is expressed as the ratio of the SA of plasma to liver PLP, and is represented by the following relation,

$$RSA = SA \text{ plasma PLP/SA liver PLP}$$

The RSA calculated from SA of plasma and liver PLP as a %ID, therefore, is used as a measure of plasma PLP turnover (23).

DATA AND RESULTS

Total Liver Lipids and Plasma Phospholipid Levels

Total Liver Lipids. Total liver lipids increased to about one and one half times control values as early as 4 1/2 hr after a single injection of *dl*-ethionine and continued to rise up to and including the 12th hour after ethionine administration (Table I). The control values for nonethionine animals were in the range of 4.96 to 6.66 g%, while the increase in total liver lipids ranged from 11.74 and 11.28 g% at 9 and 12 hr to maximum values of 13.47 and 18.60 g% 24 hr after ethionine in female animals (Tables I and II). Ethionine did not bring about an increase in total liver lipids in male rats except for a slight rise in the 24 hr postethionine period (Table II).

At 24 hr after a single administration of *dl*-ethionine there appears to be a slight difference in the extent of total liver lipid accumulation in female rats fed at the time of ethionine administration (18.60 g%) compared to the female rats that were prefasted at the time of ethionine administration (13.47 g%). Some consideration of these data is mentioned in the discussion.

Plasma Phospholipids. Plasma phospholipids were found to decrease with increasing time after ethionine to a greater extent than the decrease which was caused by fasting alone. In male rats only a slight lowering of plasma PL was observed following ethionine administration (Table II). The extent of the decrease in plasma PL in female rats given ethionine appears to be greater in animals that were fed up to the time of the ethionine administration, however the data are not conclusive on this point because of the limited numbers of animals. Further importance of these findings is discussed in connection with the specific activity of plasma phospholipids after radiophosphate administration.

Liver Phospholipids. A decrease in liver phospholipids is seen in female rats given a

TABLE III

Effect of *dl*-Ethionine on the Three Hour Uptake of $NaH_2P^{32}O_4$ Into Liver and Plasma Phospholipids in Male and Female Rats

Expt. no.	Time fasted	Time after ethionine	Sex	No. of rats	Liver phospholipids		Plasma phospholipids		Relative specific activity ± SD
					% ID[a] ± SD[b]	SA[c] ± SD[b]	% ID ± SD	SA ± SD	
	(hr)	(hr)			(%)		(% x 10^{-1})		
III	24	0	F	2	.83 ± .07	.63 ± .12	.21 ± .02	.65 ± .08	1.02 ± .06
	48	0	F	2	.74 ± .02	.62 ± .09	.24 ± .01	.58 ± .01	0.94 ± .15
	24	6	F	2	.25 ± .04	.21 ± .02	.02 ± .01	.09 ± .05	0.44 ± .20
	48	24	F	2	.48 ± .07	.50 ± .07	.04 ± .02	.22 ± .10	0.41 ± .13
	48	24	M	3	.32 ± .02	.26 ± .01	.04 ± .01	.26 ± .08	0.99 ± .35
IV	3	0	F	3	.50 ± .13	.43 ± .09	.09 ± .03	.15 ± .05	0.33 ± .07
	6	0	F	2	.69 ± .03	.65 ± .03	.11 ± .10	.19 ± .16	0.29 ± .25
	24	0	F	2	.95 ± .06	.91 ± .09	.22 ± .06	.37 ± .13	0.41 ± .18
	3	3	F	3	.38 ± .07	.33 ± .06	.03 ± 0	.06 ± .00(1)	0.17 ± .03
	6	6	F	2	.46 ± .09	.41 ± .08	.07 ± .01	.15 ± .03	0.35 ± .01
	24	24	F	2	.70 ± .10	.84 ± .19	.13 ± .06	.79 ± .45	0.91 ± .33
V	3	0	M	3	.36 ± .12	.31 ± .07	.05 ± .01	.09 ± .02	0.28 ± .03
	6	0	M	2	.21 ± .03	.20 ± .05	.05 ± .01	.07 ± .00(2)	0.36 ± .11
	24	0	M	2	.64 ± .09	.55 ± .05	.12 ± .02	.31 ± .07	0.55 ± .07
	3	3	M	3	.13 ± .02	.13 ± .02	.01 ± 0	.01 ± 0	0.15 ± .04
	6	6	M	2	.33 ± .02	.27 ± .02	.05 ± .03	.11 ± .03	0.38 ± .08
	24	24	M	2	.36 ± .08	.32 ± .04	.06 ± 0	.18 ± .03	0.58 ± .02

[a]ID, counts per minute as a per cent of injected dose.

[b]SD, ± Standard Deviation.

[c]SA, specific activity of PLP.

single injection of *dl*-ethionine. There were no significant differences in liver phospholipid levels in ethionine-injected male rats (Tables I and II).

The range of liver PLP was from 1.15 to 1.43 mg-P/g liver in female rats in the fed and fasted conditions. At 6 and 24 hr after ethionine, the values ranged from 0.85 to 1.06 mg-P/g liver.

Phospholipid Labeling Experiments

Liver. The per cent of injected radiophosphate incorporated into phospholipids per gram of liver was less in both male and female rats given ethionine than it was in control rats which had only been fasted for the corresponding periods of time (Table III). This decrease was observed as early as 3 and 6 hr after ethionine administration. After longer postethionine periods, the per cent ID rose above the 3 and 6 hr values, but the values were still below those for the nonethionine, fasted control rats.

The SA of liver PLP was lower in the ethionine treated rats than in their controls. Fasting by itself caused some increase in the SA of liver PLP in both male and female rats. It is especially noteworthy that the SA of liver PLP was lower in ethionine-treated rats than in their corresponding controls even though the amounts of liver PLP decreased considerably after ethionine administration.

Plasma. The per cent injected radiophosphate incorporated into phospholipids per milliliter of plasma was much less in both male and female rats given ethionine than it was in control rats which had only been fasted for the corresponding periods of time (Table III). The decrease, amounting to as much as one tenth of the control values, was observed as early as 3 and 6 hr after ethionine administration. Following the trend in the liver, the per cent ID was elevated somewhat at the longer postethionine intervals, but the values were still lower than the corresponding control values. Fasting by itself caused some increase in the per cent ID of plasma PL in both male and female rats.

The SA of plasma PLP was lower in the ethionine-treated rats than in their controls except in the fed female rats 24 hr after ethionine. Fasting by itself caused some increase in the SA of plasma PLP in both male and female rats. The higher SA of plasma PLP in fed female rats 24 hr after ethionine can be accounted for, in part, by the decrease in total plasma PL at this time. As in the case of the liver, it is noteworthy that the SA of plasma PLP was lower in ethionine-treated rats than in their corresponding controls even though the amounts of plasma PLP were decreased considerably by ethionine.

Relative Specific Activity (RSA). The decrease in the SA of PLP brought about by ethionine was greater in plasma than in liver at early time intervals after ethionine. Calculation of the RSA shows that ethionine reduced the values to about one half the control values 3 hr after the drug administration. Some increase in the RSA was observed at 6 hr after ethionine administration. After prolonged fasting, and at the 24 hr interval after ethionine, there was a tendency for the RSA to increase considerably, approaching unity.

Thus, it appeared that there was a decrease in the appearance of newly-labeled phospholipids in the plasma immediately following ethionine administration, i.e., at 3 hr, and that there was a subsequent increase at longer postethionine intervals, namely 6 and 24 hr. This seems to indicate a remobilization under these conditions not unlike the increased mobilization of lipids occurring after fasting. Correspondingly, in the fed animals, the RSA of plasma to liver PLP is very low, indicating, in both males and females, that the liver is mobilizing relatively small amounts of phospholipid to the plasma.

Since there is a significant difference in the RSA found for 24 hr fasted female control rats in Experiment III (1.02 ± 0.06) and in Experiment IV (0.41 ± 0.18) differences in the age, weight and previous nutritional history of the animals in these two groups would appear to have important bearing on these data. Uniformity of the animals was maintained in each of the experimental groups, but this did not insure uniformity between groups. Hence, the data are more relevant to the differences found at the several time intervals after ethionine, and after fasting, than to the other conditions of the experiments.

DISCUSSION

The inhibition of liver and plasma phospholipid synthesis, as measured by the 3 hr uptake of labeled orthophosphate, can be related to the action of ethionine as an antimetabolite of methionine (10,11). The decreased availability of choline brought about by ethionine administration would by itself account for the decreased formation of phospholipids seen in this study. The decrease in the synthesis of phospholipids was greatest at the early intervals after ethionine. Since ethionine is very rapidly excreted after administration (12,13), it is possible that the effects of ethionine on phospho-

lipid synthesis are operant only when the level of circulating ethionine is high. Certainly the blocking effects would be greatest during this time. Important in this regard is the fact that ethionine was administered as a single dose rather than in multiple doses as in some previous studies (10,11,14).

It is important to note that most of the circulating lipids of plasma are present as lipoproteins (7,15) and that liver PLP is regarded as the immediate precursor of the plasma PLP (16,17) which is principally choline-containing phospholipid (18). Rees and Schotlander (19) have shown that serum NEFA increases, and serum PL decreases in both male and female rats at 6,13 and 24 hr after the administration of *dl*-ethionine, while liver PL show little or no change and total liver lipids increase markedly. Olmsted (20) made similar observations on these parameters, except NEFA, and showed further that the liver glycogen was reduced to a trace and blood glucose fell to values below 20 mg%. Dole (21) demonstrated a relation between feeding glucose and decreased NEFA levels in serum. While the increase in serum NEFA brought about by ethionine (19) or brought about by the low glucose after ethionine (20), may enhance the availability of NEFA as a substrate for liver lipoprotein formation, ethionine also blocks the formation of phospholipids and, therefore, faulty mobilization of lipoprotein would account for the rapid increase in liver triglyceride under these conditions.

The condition of a rapidly increasing liver triglyceride content brought about by the single administration of *dl*-ethionine was accompanied in the present study by a decrease in the concentration of phospholipids in the liver and plasma. Inspite of these decreased levels of PL, the SA of liver and plasma PLP was lower after ethionine. Because the SA of plasma PLP decreased more than the SA of liver PLP at the early time intervals after ethionine, the relative specific activity (RSA) decreased also indicating a decreased transport of liver PL to the plasma (22,23). Some notable exceptions to these combined findings are important to consider. These are the increase in the RSA at later time intervals after ethionine administration and during fasting and, in addition, the changes in ethionine-treated male rats which did not get a fatty liver. Changes in all of these conditions are consistent with the view that increased phospholipid transport from the liver is associated with increased mobilization of lipids through the triglyceride cycle. Work by Zilversmit et al. shows increased RSA for PLP under conditions where the uptake of radiophosphate into plasma and liver PL is increased such as following pancreatectomy (23) and after phlorizin poisoning (24).

The data presented here are adequate to make conclusions regarding the SA of liver and plasma PLP and the RSA of these as a function of time after ethionine administration, and as a function of time after fasting. That is, the conditions were uniform within each of the experiments and a time-course of events after the single administration of ethionine can be followed. Further, these events are consistent in each of the experiments regarding the effects of ethionine and fasting on these parameters. However, it would not be justifiable to compare the fasted females with the fed females, nor the males with the females, unless replicate experiments were done under each of these conditions. Thus, the fact that there is a marked variation in the RSA value for the 24 hr fasted female rats in Experiments III and IV points out the importance of uniformity of previous nutritional history of the animals on the results of these experiments. Since there was uniformity in this regard within each of these experiments, the major conclusions are based on the changes as a function of time after fasting and after ethionine administration in each of the individual experiments.

The concept of remobilization of liver PL following a period of inhibition, and the increased mobilization of liver PL during fasting suggests a role for phospholipids in the triglyceride cycle. Zilversmit has shown that the ratio of specific activities of plasma to liver PLP is a better measure of plasma PL turnover than the SA of plasma PLP as a function of time or even the ratio of plasma PLP to plasma acid soluble phosphorus specific activities, since the conversion of radiophosphate into liver PLP could be changed without altering the release of phospholipids to the plasma (23).

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[Received August 14, 1968]

Studies on the Role of Phospholipids in the Triglyceride Cycle: II. Turnover of Plasma Phospholipids in Ethionine-Treated Dogs[1]

C. A. OLMSTED, Department of Biological Sciences, Louisiana State University in New Orleans, Lake Front, New Orleans, Louisiana 70122

ABSTRACT

The disappearance of native labeled plasma phospholipids ($nPLP^{32}$) was abruptly halted in the plasma of normal mongrel dogs shortly after giving *dl*-ethionine by intraperitoneal injection. In other dogs, chronic feeding of ethionine over a period of three days prior to these measurements prolonged the turnover time of plasma phospholipids. This impairment in the turnover of plasma PL brought about by ethionine administration occurs regardless of sex differences in lipid metabolism or other physiological differences relating to the nutritional status of the animal. The amount of $nPLP^{32}$ found in the liver at the end of the turnover experiments was also measured. These data suggest that phospholipid transport from plasma to liver is not impaired by ethionine. The possible significance of an ethionine-induced choline deficiency which impairs transport of triglycerides and phospholipids from the liver to plasma is considered in regard to the role of phospholipids in the triglyceride cycle.

INTRODUCTION

The findings of the preceeding paper (1) suggest that increased mobilization of liver phospholipids takes place under conditions favoring transport of lipids via the triglyceride cycle such as after fasting and after or during recovery from inhibition of phospholipid synthesis by ethionine. Ethionine is known to block choline synthesis and to decrease protein synthesis in the liver (10). Even though ethionine blocked the synthesis of liver and plasma phospholipids, turnover measurements indicated that the transport of liver PL to plasma was only temporarily blocked after a single administration of *dl*-ethionine. The mechanism responsible for the release of liver PL to the plasma is unknown but may be dependent upon the levels of circulating NEFA, or glucose, or both, or upon the availability of choline in the liver for the synthesis of specific lecithins.

Since the turnover of plasma phospholipids appeared to be initially depressed by ethionine in the rat, it became of importance to investigate the effects of ethionine on the turnover of plasma phospholipids by a direct method of measurement. The disappearance of labeled, native plasma phospholipids ($nPLP^{32}$) was therefore measured in male and female dogs before and after the administration of *dl*-ethionine.

EXPERIMENTAL PROCEDURES

Animal Treatment and Ethionine Administration

Animals and Diet. Normal, healthy mongrel dogs were used throughout this investigation and were fed daily with a balanced ration of dry, kibbled dog food (Purina Dog Chow) and water.

Ethionine Administration. Dosages of *dl*-ethionine, 1 g/kg body weight, were given either orally in gelatin capsules embedded in a small amount of raw meat, or by intraperitoneal injection through a 13 G needle in a slurry with warm water. At autopsy, at the times indicated by the data, following intraperitoneal administration of ethionine, the peritoneal fluid appeared to be in excess of normal amounts probably as a result of the osmotic gradient established by the presence of the ethionine.

Experimental Design

A total of eight mongrel dogs were used in two experiments. The turnover time and turnover rate of plasma phospholipid (PL) was calculated from the disappearance of intravenously injected $nPLP^{32}$ (3-5). The effect of chronic feeding of ethionine for three days was determined by plasma PL turnover measurements in two female and two male dogs in Experiment 1, in which one female and one male dog served as nonethionine controls. In Experiment 2, the effect of acute (single) ethionine administration on plasma PL turnover was measured in two female and in two male dogs; in this experiment each dog served as its own control with several measurements before and after ethionine administration.

[1]Presented in part at the 58th Annual AOCS Meeting, New Orleans 1967.

TABLE I

Turnover of Plasma PLP^{32} After Chronic Ethionine Feeding

Dog	Sex	Ethionine dosage	Plasma volume		Turnover time (t_t)	Total liver lipids	S. A. of liver PLP as % ID
		(g/day)	(ml)	(% BW)[a]	(hr)	(g %)	
1	F	1.0	242	5.5	11.9	11.2	0.100
2	F	0	468	4.7	7.5	5.6	0.047
3	M	1.0	409	5.7	52.8	5.8	0.071
4	M	0	512	5.2	10.0	6.9	0.057

[a]% BW, Total plasma volume as a per cent of body weight.

Labeling of Plasma Phospholipids

Two normal female dogs were used as donors for $nPLP^{32}$. Each donor was injected intraperitoneally with 2 mC of P^{32}-orthophosphate ($NaH_2P^{32}O_4$) in 1.0 ml of 0.154 M NaCl. Twenty-four hours after radiophosphate administration the dogs were lightly anesthesized with pentobarbital, and blood was withdrawn completely from the femoral artery. After separating the oxalated plasma by centrifugation, the plasma, containing nonpurified $nPLP^{32}$, was injected intravenously into the recipient experimental dogs without further treatment since it was shown (2) that the activity remaining in the acid-soluble fraction under similar conditions did not contribute significantly to the total activity by synthesis to phospholipid P^{32} of liver and plasma.

Injection of $nPLP^{32}$ and Tissue Sampling

To measure the turnover of plasma PL, recipient dogs received 40-55 ml of whole plasma containing $nPLP^{32}$ presumed to be in its native state, i.e., complexed with lipoproteins. This was injected intravenously into an exposed femoral vein under pentobarbital anesthesia. At various times after the injection of $nPLP^{32}$ 5 ml samples of arterial blood were withdrawn for analysis. Liver samples were obtained at the termination of the experiments.

Chemical and Radiochemical Analyses

Total liver lipids and liver phospholipids were extracted and measured as previously described (1), as were the analyses of phospholipid P^{32} (PLP^{32}) and phospholipid phosphorus (PLP^{31}). Phospholipids were separated from the plasma by using trichloracetic acid precipitation as described by Zilversmit and Davis (6). The precipitates, in which all of the phosphorus is PLP, were dissolved in a few drops of 10% NaOH and suitable aliquots were taken for the determination of PLP^{32} and PLP^{31}. Plasma PL was calculated as PLP^{31} x 25 and expressed as mg %.

Calculation of Turnover Time and Turnover Rate

Turnover Time. The turnover time (t_t) of plasma PL is expressed in minutes and was calculated from the relationship set forth by Zilversmit (3):

$$t_t = 1.44\ t_{1/2}$$

where the half life ($t_{1/2}$) was determined from a plot of log cpm/ml plasma PLP^{32} as a function of time following intravenous injection of $nPLP^{32}$.

Turnover Rate. The turnover rate (p) of plasma PL was calculated from the relationship set forth by Zilversmit (3):

$$p = r/t_t$$

where r is the PL concentration in the total circulating fluid volume (F) which was calculated by extrapolation of the curve of log cpm/ml of plasma PLP^{32} back to zero time (t_o). By the method of isotope dilution,

$$F = (x/f)t_o/X_i$$

where X_i is the total $nPLP^{32}$ activity injected at t_o, and where (x/f) is the amount of PLP^{32} cpm/ml at any time, t. Therefore, $(x/f)t_o$ is the amount of $nPLP^{32}$ at the extrapolated zero time, and hence,

$$r = F \cdot (\text{mg PL/ml})$$

It has been shown that plasma $nPLP^{32}$ exchange with the red blood cells is negligible (7), and the values for plasma volume so obtained in this study were in agreement with values obtained by dye dilution (8) and by tagged red cell methods (9).

DATA AND RESULTS

Chronic Feeding of dl-Ethionine

Two dogs fed ethionine for three days prior to the measurement of the disappearance of intravenously injected $nPLP^{32}$ showed an increased turnover time with values ranging from 4.4 to 42 hr greater than the corresponding control values (Table I). It should be

TABLE II

Turnover of Plasma PLP^{32} Before and After *dl*-Ethionine Administration

Dog	Sex	Plasma volume		Time after ethionine	Turnover time (t_t)	Turnover rate (p)	Total liver lipids	S.A. of liver PLP as % ID
		(ml)	%BW[a]	(min)	(min)	(mg/hr)	(g %)	
1	F	322	7.3	0	225	6.85	---	---
		---	---	43	∞	0	4.86	0.067
2	F	564	6.9	0	239	14.90	---	---
		---	---	305	∞	0	5.29	0.121
3	M	448	6.4	0	214	14.30	---	---
		---	---	125	2190	0.02	4.95	0.052
4	M	584	5.6	0	214	11.70	---	--
		---	---	299	∞	0	5.96	0.046

[a]%BW, Total plasma volume as a per cent of body weight.

pointed out, since only two female and two male dogs were used in this study, that the only conclusion to be made concerns the prolonged turnover time of plasma phospholipid following ethionine administration. Although a significant increase in total liver lipids is observed in the female dog fed ethionine, it would not be justifiable to make further conclusions regarding other differences seen here in the male and female dogs. The specific activity (SA) of liver PLP measured at the end of this experiment, is considered in the discussion.

Single Administration of dl-Ethionine

In both male and female dogs given a single intraperitoneal injection of *dl*-ethionine there was an abrupt cessation of the disappearance of intravenously injected $nPLP^{32}$. The turnover time calculated from this data was between 3½ and 4 hr (214 to 239 min) in all four dogs prior to the administration of ethionine; correspondingly, the turnover rate varied from 6.8 to 14.9 mg PL/hr. The turnover time after ethionine increased to 2190 min in one dog to an infinitely long period in the three other dogs; correspondingly, the turnover rate decreased to 0.02 mg PL/hr in one dog and was zero in the other three dogs. Total liver lipids were not found to be unusually high even 5 hr after the ethionine administration (Table II).

It is important to note that the decreased PLP^{32} cpm as a per cent of the injected dose (% ID) showed an abrupt cessation immediately after the administration of ethionine. In addition, there was a tendency for the PLP^{32} cpm as % ID to increase somewhat during the first few measurements after ethionine. In none of the measurements after ethionine was the PLP^{32} cpm as % ID lower than pre-ethionine measurements. The SA of liver PLP measured at the end of this experiment is considered in the discussion.

DISCUSSION

The turnover time of plasma PL was prolonged in two dogs when measured three days after daily ethionine feeding compared to two other dogs which had not been fed ethionine. However, since ethionine feeding and related nutritional conditions alter the physiological state of the animal in a variety of ways over a period of several days, it would seem that more data would be required to establish the nature of any differences in turnover time prolongation seen in these two animals. These conditions are obviated in the other experiments in which the acute effects of a single administration of *dl*-ethionine were observed during short time intervals following ethionine administration since each of the four animals served as their own controls, with several measurements made before and after the administration of ethionine. From these data there is little doubt that the turnover of plasma PL is impaired by the administration of *dl*-ethionine, and that this occurs regardless of sex differences in lipid metabolism or other physiological differences relating to the nutritional status of the animal.

Immediately after the intraperitoneal administration of ethionine there was a tendency for the PLP^{32} cpm as % ID to increase somewhat during the time of the first few measurements. It is believed that this may have occurred as a result of some hemoconcentration brought about by the high concentration of ethionine in the peritoneal cavity. It was noted that the peritoneal fluid seemed to be in excess at the termination of these experiments. One dog (female dog 1, Table II) in which only three post-ethionine measurements were made, died 43 min after ethionine administration due to hemorrhage and this effect was not seen in that animal. The data in Figure 1 show that the PLP^{32} cpm as %ID leveled off afterwards, and that in no instance was the

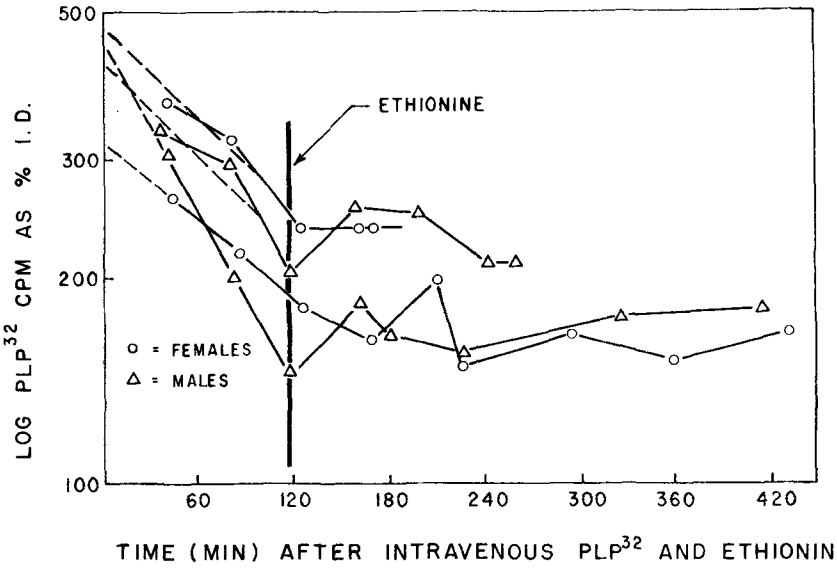

FIG. 1. The disappearance of nPLP32 from plasma of male and female dogs before and after the administration of *dl*-ethionine. The broken lines (—) represent an approximation of the zero time extrapolations for log cpm of PLP32 as %ID used for the measurement of total circulating plasma volume.

PLP32 cpm as %ID lower than pre-ethionine values. The abrupt cessation of the utilization of plasma PL following ethionine administration is apparent in every case.

The female dog fed ethionine for three days accumulated more total liver lipid than its control and more than either the male control dog or the male dog fed ethionine. In male and female dogs given a single injection of ethionine, the total liver lipid content was not unusually high even 5 hr after ethionine administration. A greater amount of nPLP32 was taken up by the livers of the two animals fed ethionine than by their controls. Similar data are presented for this measurement after the single, acute, administration of ethionine, but not all of the data are comparable because of the differing times after nPLP32 injection. It does appear, however, at 5 hr after ethionine (Table II, dog 2 and dog 4) that the liver of the female dog took up more nPLP32 than the liver of the male dog. Although more data would be desirable regarding these points, the present findings suggest increased uptake of nPLP32 by the livers of ethionine-treated animals and shows that this occurred to a greater extent in females than in males in both of the experiments presented here (Tables I and II). Such appearance of greater amounts of nPLP32 in the liver might be due to a greater uptake, or a decreased utilization, or both. The data of the preceeding paper (1) indicate that mobilization from the liver to plasma is initially decreased and then is increased at later time intervals after ethionine administration, and that this may be a function of circulating ethionine concentration. That an increased exchange of liver and plasma PL is related to accumulation of liver triglyceride and to the cyclic events of lipid mobilization is suggested by such findings. Thus it would appear that conditions favoring increased hepatic triglyceride turnover might act as a stimulus to mobilization of liver phospholipid, but further work is needed to firmly establish this point.

It is not unreasonable to believe that phospholipids participate in the redistribution of fat. Phospholipids carried by the blood exist as part of the lipoprotein complex. The data of this and the previous study strongly suggest that the inability of the ethionine-treated animal to mobilize this lipid from the liver to the plasma and utilize the lipid peripherally results in its accumulation by the liver.

The fact that ethionine stopped the disappearance of nPLP32 abruptly would indicate that some immediate effect of ethionine was responsible for the lack of phospholipid mobilization. The decreased cellular levels of choline immediately following ethionine administration might account for this occurrence, and since ethionine blocks the choline synthesis which is required for plasma phospholipid synthesis and mobilization from the liver to the plasma, one

might ask the question: "Is choline essential for the cellular transfer mechanisms of phospholipid transport?" Olivecrona (11) calculates that all of the triglyceride appearing in the liver following ethionine administration could come from the NEFA of plasma, thus supporting the hypothesis that the increased mobilization of lipid to the liver during the time when phospholipid synthesis is depressed results in the accumulation of liver during the time when phospholipid synthesis is depressed results in the accumulation of liver TG because of the inability of the liver to mobilize lipoproteins, the latter being dependent upon de novo PL synthesis.

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[Received August 14, 1968]

Endocrine Control of Fat Mobilization in the Isolated Fat Cells of Cold-Exposed Rats

DONALD G. THERRIAULT, ROGER W. HUBBARD and **DONALD B. MELLIN,**
Biochemistry-Pharmacology Laboratory, U.S. Army Research Institute
of Environmental Medicine, Natick, Massachusetts 01760

ABSTRACT

Adipose tissue in rats maintained at normal ambient temperature grows by a mixture of cell proliferation and lipid deposition in the early growth stage of the rat. In the mature rat, the tissue grows primarily by lipid deposition, mitotic activity being significantly decreased. When the rat is acclimated to 5 C, growth of adipose tissue is less than that of rats maintained at normal ambient temperature. Furthermore, growth of adipose tissue in the 5 C rat occurs through a mixture of cell proliferation and lipid deposition throughout the body weight range studied. The differences in tissue growth were taken into consideration in measuring the stimulatory effect of norepinephrine on lipolysis and reesterification of isolated fat cells. The results indicate that the cell size affects the lipolytic response; the larger the cell the less sensitive it is. Fat cells from cold-acclimated rats are more sensitive to the lipolytic action of norepinephrine, independent of differences in cell size. On the other hand, reesterification is not affected by cell size, nor by exposure of the rat to cold

INTRODUCTION

It has been shown by several investigators that the metabolic activity of adipose tissue is affected by the age of the rat as well as by his previous environmental history. Benjamin et al. (1) reported that the in vitro rates of acetate and palmitate incorporation into the mixed lipids by adipose tissue, as well as the rate of free fatty acid release under the stimulus of epinephrine, decreased with increasing age. Gries and Steinke (2) studied the in vitro metabolism of 1-^{14}C-glucose by adipose tissue into $^{14}CO_2$ and total ^{14}C lipids, employing both adipose tissue segments and isolated fat cells. They found that the basal glucose metabolism and response to insulin decreased with increasing body weight of the animal. There is also evidence to suggest that the lipolysis system in adipose tissue is more active in cold-acclimated rats than in animals maintained at normal ambient temperature. Hannon and Larson (3) have shown that the epididymal fat tissue from cold-acclimated rats is considerably more sensitive to the free fatty acid mobilizing action of norepinephrine than is similar tissue from control rats. Mitchell and Longwell (4) also found that tissue from cold-acclimated rats released more than three or four times the amount of free fatty acids during incubation than did tissues from rats acclimated to a warmer environment.

The question raised by these experiments is whether the results represent a true change in metabolic activity of the tissue or are due to an artifact resulting from some other phenomenon. Recent studies by Salans et al. (5) indicate that the cellularity of the tissue sample is of prime importance in evaluating the metabolic activity of adipose tissue. They studied the incorporation of glucose into CO_2 and triglycerides by adipose tissue from individuals with adipose cells of widely different sizes. When the activity was expressed per unit of lipid, the various functions proceeded at different rates in these tissues. However, when activity was expressed per cell, these observed differences disappeared and tissues with cells of widely different sizes metabolized glucose at similar rates per cell. In contrast, the insulin responsiveness of the tissue was found to be closely related to the size of the adipose cells in the tissue fragments. The larger the mean cell size in a tissue sample, the less responsive it was to insulin. Since the previous authors expressed metabolic activity per unit weight of adipose tissue, it is not possible to determine whether the change in activity which they report was due to differences in cell size, number or cell function.

Of the several aspects of tissue physiology yet to be elucidated is the extent to which age will affect the number and size of fat cells in adipose tissue. Nor is it known how chronic cold exposure affects growth of adipose tissue. Though it is generally concluded that a low body fat content is an inevitable consequence of prolonged exposure to cold (6-8), it is not known whether this is due to a decreased deposition of fat within the tissue or a decreased tissue growth. In order to properly evaluate the effect that the growth of the animal, or its acclimation to a cold environment, may have on the metabolic activity of adipose tissue, it is essential to understand how these conditions affect growth of the tissue. It is the purpose of this investigation to follow changes within adi-

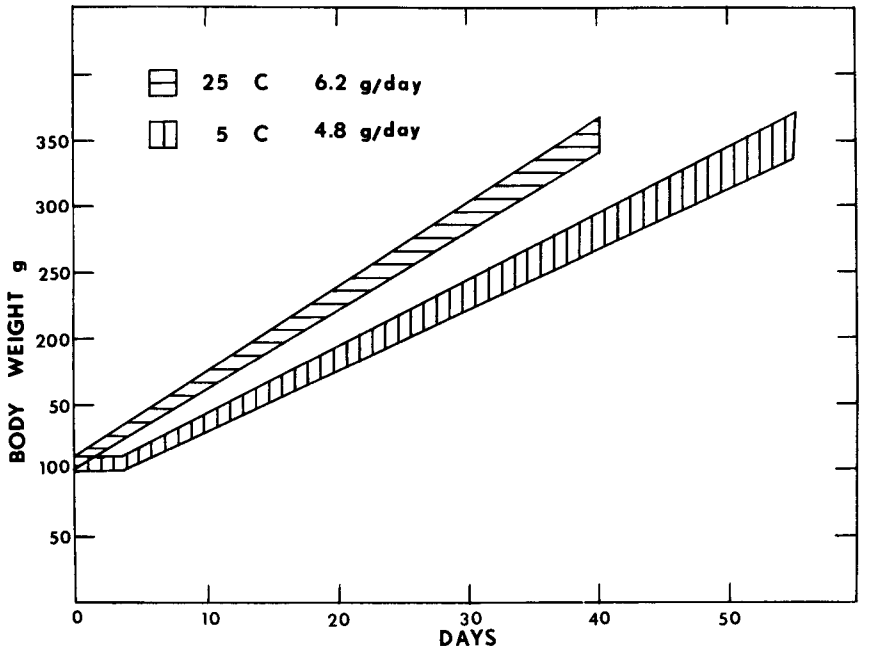

FIG. 1. Growth curve for rats kept either at 25 C or 5 C. The area within the individual curves includes the values for 95% of the population. There were 60 rats in each group. The rats were weighed each day for the first five days and then twice per week thereafter.

pose tissue taking place during growth. This was done in rats maintained at normal ambient temperature or acclimated to 5 C. Since adipose tissue is diffusely dispersed throughout the organism, it does not lend itself readily to quantitative isolation. For that reason, the present investigation was limited to a study of the epididymal fat pad. It has specific and discrete boundaries throughout all ranges of enlargement, thus rendering its quantitative removal possible at different ages and degrees of fat accumulation. In order to generalize from these results, it is necessary to assume that the epididymal fat pad is representative, at least qualitatively, of all white adipose tissue.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 50-75 g were individually caged in one of two environmental chambers and maintained at either 25 ± 2 C or 5 ± 1 C. Each group of rats was weighed each day for the first five days and then twice per week thereafter. Animals were kept in the cold chamber at least 6 weeks and up to 14 weeks. The control animals at 25 C were killed at intervals from 1 week to 12 weeks after being placed in the environmental chamber. They had free access to tap water and Purina Chow until they were killed by decapitation. Both epididymal fat pads were immediately excised, blotted and weighed. One pad was used for tissue lipid analysis while the contralateral pad served for the preparation of isolated fat cells.

Preparation of Isolated Fat Cells

Isolated fat cells were prepared by a modification of Rodbell's (9) procedure. Each gram of tissue was added to 6 mm of Krebs-Ringer phosphate buffer, pH 7.4, prepared without calcium, containing 4% bovine serum albumin and 3 mg/ml bacterial collagenase (Worthington Biochemical Co.). The mixture was incubated at 37 C in a shaking water bath for 75 min. The mixture was then strained through a plastic mesh into a clean polyethylene vial and incubated an additional 15 min to insure uniformity in cellular disaggregation. The fat cells were floated to the surface by centrifugation in an International Clinical Centrifuge, Model CL, for 30 sec at 600 rpm. The sediment and infranatant solution were removed by aspiration and the cells washed with Krebs-Ringer phosphate buffer containing 4% albumin.

Histological examination of the cells after staining with methylene blue revealed the presence of mainly unilocular fat cells. The recovery of cells from the intact tissue, based

TABLE I

Effect of Environmental Temperature on Epididymal Fat Pad Lipid Content[a]

Weight	25 C	5 C
Rat weight, g	420 ± 14[b]	423 ± 8
Epididymal fat pad weight, g	4.81 ± 0.21	3.06 ± 0.24
Gram triglyceride per 100 g body weight	1.04 ± 0.11	0.64 ± 0.08

[a]Rats were maintained at 5 C for eight weeks.

[b]There were eight rats in each group. Values are the means ± S. E.

on triglyceride analysis of the tissue and cells, was 87.4 ± 1.8%.

Lipid Analysis

The intact tissue was homogenized in a glass tissue grinder with methanol. Sufficient chloroform was then added to make the solvent mixture chloroform-methanol (2:1 v/v). The mixture was further homogenized and filtered through a glass fiber filter pad using positive nitrogen pressure. Extraction of the residue was repeated three separate times with chloroform-methanol (2:1). The lipid extracts were combined in a round bottom flask, and the solvent removed by rotary evaporation in vacuo at 15 C. The residue remaining after evaporation of the solvent was dissolved in 10 ml chloroform. The chloroform solution was washed with H_2O to remove nonlipid material, and aliquots of the washed chloroform solution used for gravimetric determination of lipid.

The fat cell suspensions were centrifuged and the infranatant solutions removed by aspiration. The cells were washed once with Krebs-Ringer phosphate buffer, centrifuged and the buffer removed. The cells were quantitatively transferred with methanol into 15 ml glass-stoppered centrifuge tubes. Extraction of the lipid was carried out as described above with the exception that the lipid extract was recovered by centrifugation and decantation.

DNA Determination

The pellet containing the DNA from the lipid extracted fat cells was washed with cold 2% perchloric acid and centrifuged. The supernatant was discarded and the pellet resuspended in 2.0 ml 2% perchloric acid, capped with aluminum foil and heated in a water bath at 90 C for 45 min. The tube was allowed to cool, centrifuged, and the supernatant containing the DNA decanted into a clean glass-stoppered centrifuge tube. The remaining pellet was extracted again and the extracts combined. DNA was determined spectrophotometrically after reaction with indole according to Ceriotti (10).

Lipolysis and Reesterification

Lipolysis and reesterification were determined simultaneously with the fat cell suspension using the balance method described by Vaughan (11). The fat cells containing between 1-3.5 mg DNA were incubated in 4 ml Krebs-Ringer phosphate buffer pH 7.4 (without calcium) containing 4% albumin, 1 mg/ml glucose and 3 nmoles/ml norepinephrine. Incubation was carried out at 37 C in a shaking water bath. After 30 min the reaction was stopped by the addition of 5 ml Dole's acid extraction mixture (12). Appropriate zero time controls as well as blanks containing everything but fat cells were run concurrently.

The total fatty acid content of the incubation flasks was measured by the procedure of Rodbell (9). The DNA content of the cells was then calculated by multiplying the DNA-triglyceride ratio of the original cell suspension by the total triglyceride measured in each flask.

Free fatty acids in the system were separated by an alkali extraction of the hexane solution. The aqueous phase was then acidified and the fatty acids reextracted with hexane. The free fatty acids in hexane solution were determined by the titration method of Dole and Minertz (12).

The free glycerol produced by lipolysis of triglycerides was measured fluorometrically, using a micromodification of the enzymatic method of Vaughan (11), in which NADH formation was measured with an Aminco-Bowman spectrophotofluorometer. The wave length of the incident light was 360 mμ and the transmitted light was measured at 465 mμ.

RESULTS AND DISCUSSION

When the rat is exposed to a cold environment, it must maintain body temperature. This is accomplished by an immediate increase in metabolic rate (13,14) which is accompanied by an increased mobilization of free fatty acids (15,16). However, this increased caloric expenditure is not accompanied by an increased caloric intake, consequently a normally growing

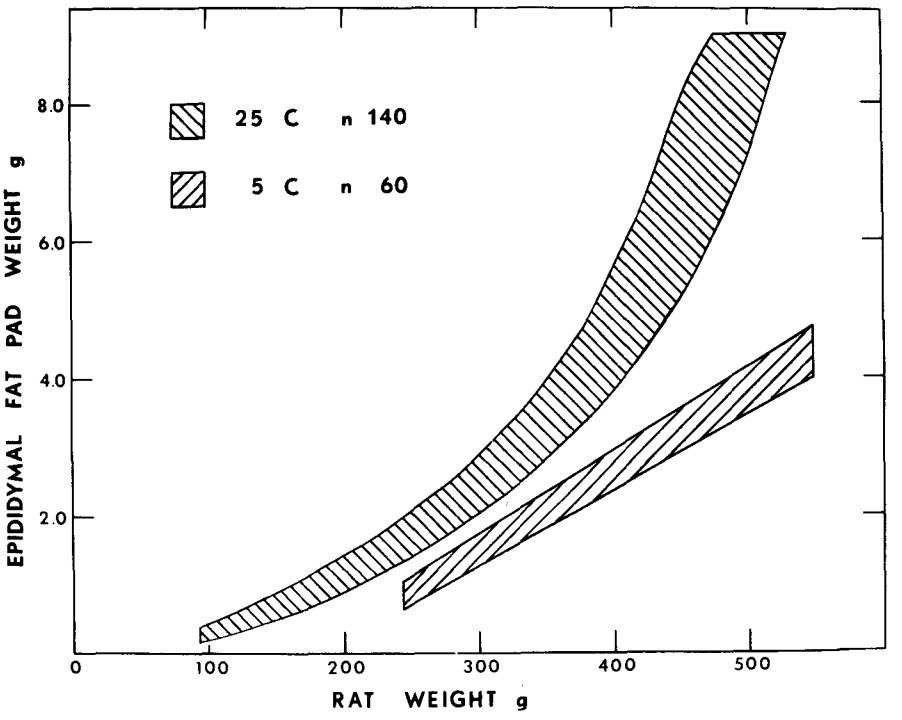

FIG. 2. Epididymal fat pad mass as a function of rat weight. The area within the individual curves includes the values for 95% of the population.

animal will cease to grow (Fig. 1). As time progresses in the cold, the animal does increase his food intake and growth is resumed (7). It can be seen in Figure 1 that even with an almost doubling of food intake, the rate of growth in the cold is considerably less than that of animals maintained at normal ambient temperature. The decrease in growth in cold-acclimated rats is due, at least in part, to a lesser amount of adipose tissue as is illustrated in Table I. Care was taken to compare animals of similar weight. This required that the groups be of different chronological age. The animals maintained at 5 C were older than those kept at 25 C. Even under these conditions, where one would expect the older animals to deposit more fat, rats kept in the cold had smaller epididymal fat pads than their controls at 25 C. When the amount of triglycerides in the epididymal fat pads in each group is compared, it becomes obvious that rats maintained at 5 C are leaner. These results are in agreement with those reported by Page and Babineau (7). These authors compared adipose tissue content in rats of similar chronological age, but of different weight.

Since the mass of adipose tissue is known to increase steadily during growth (17) we followed the growth of the epididymal fat pad as a function of the rat's growth at the two environmental temperatures. The results are graphically illustrated in Figure 2. In rats weighing 100-250 g, maintained at 25 C, there was a linear relationship between the weight of the epididymal fat pad and the weight of the animals. However, when the animals grew beyond this weight, there was a disproportionate growth of the fat pad. The contribution of adipose tissue to the total body weight became much greater as the rats grew larger in size. This is not true of rats acclimated to 5 C. In this group the linear relationship between the mass of epididymal fat pad and body weight was maintained over the entire range of rat weights studied. The contribution of adipose tissue to total body mass remained constant.

Enlargement of adipose tissue mass may result from either cellular proliferation, or the deposition of increasing amounts of lipid within preexisting tissue. The concept that adipose tissue undergoes cellular changes during states of excessive lipid deposition has been suggested or demonstrated by several investigators. The composition of adipose tissue has been shown to change with growth, with an increase in the proportion of lipid and a concomitant decrease

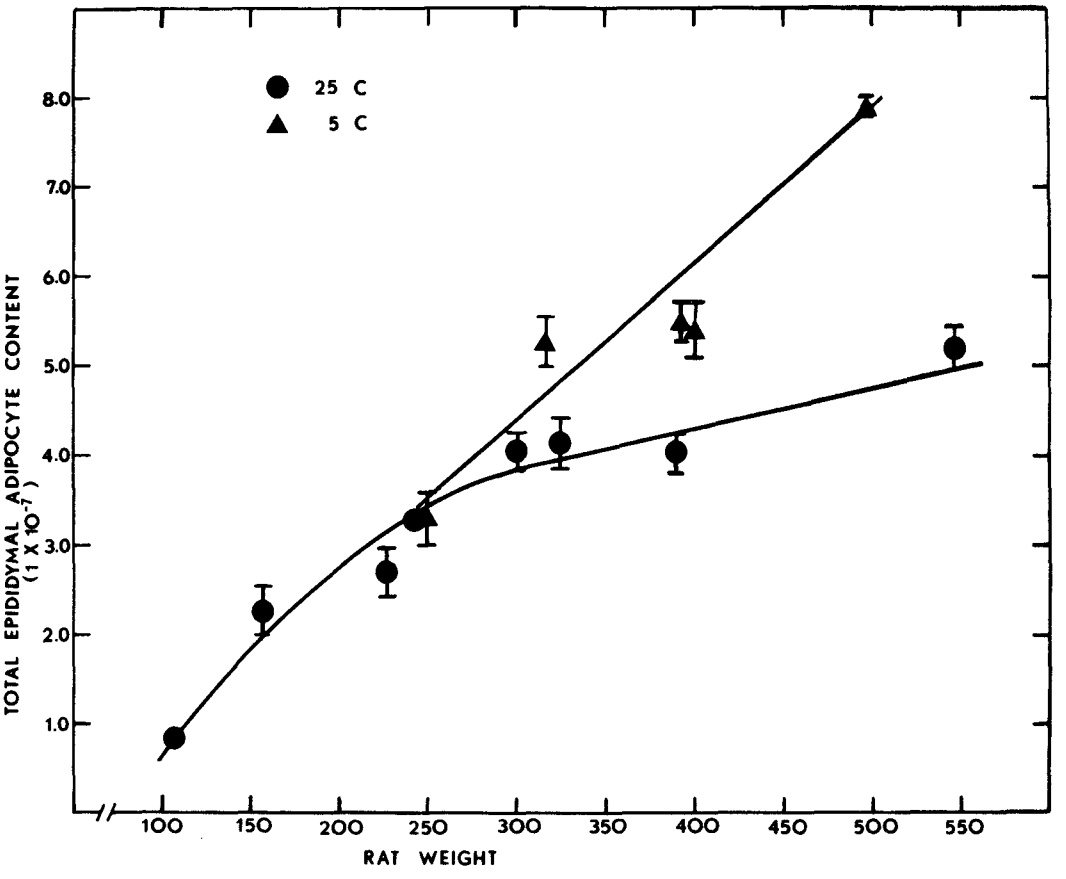

FIG. 3. Relation between total adipocyte content of the epididymal fat pads and the rats' body weight. Each point is the mean ± SEM of six separate experiments. All determinations were carried out in triplicate.

in the nitrogen content (17,18). At least two other studies have shown that the DNA content of specific fat depots in the rat increased following excessive lipid deposition (19,20). Although mitotic activity has also been demonstrated in adipose tissue utilizing the method of autoradiography (21), it remains to be demonstrated which cell type undergoes mitotic division during these states. Rodbell (9) has shown that a high proportion of the DNA content of adipose tissue can be accounted for in the stromal portion that had been separated during the preparation of isolated fat cells from epididymal fat pads. The DNA in the whole tissue was about four times greater than the amount of DNA contained in the fat cells alone. Therefore, measurement of the DNA content of the intact epididymal fat pad is inappropriate when one wishes to measure the number of fat cells in the tissue. Since we were interested not in the total cellularity of the tissue, but rather in the number and size of the fat cells in the tissue, a different approach was attempted.

It is, of course, possible to determine the DNA content of adipose cells prepared by digestion of the tissue with collagenase. Advantage was taken of the similarity of the contralateral epididymal fat pads in a given animal (20). The complete fat pads were excised and weighed. One pad was used to determine the total triglyceride content of the tissue, while isolated fat cells were prepared from the contralateral fat pad. Both DNA and triglyceride content of the isolated fat cells were determined. Assuming that all the triglyceride in the tissue was present as fat cell triglyceride, and knowing the ratio of DNA-triglyceride in fat cells, it was possible to calculate the total DNA in the intact tissue originating from the fat cells exclusively. The total DNA content of the tissue obtained in this way was used as an index of the number of fat cells. The generally accepted value of 7 x

TABLE II

Adipose Tissue Composition of Rats Maintained at 25 C or 5 C[a]

Composition	Unit of measure	25 C	5 C
Rat weight	g	326 ±24[b]	317 ±14
Cell composition			
DNA-triglyceride	μg/g	109 ±10	206 ±17
Triglyceride-cell	mg x 10^{-5}	6.43 ± 0.51	3.41 ± 0.35
Tissue composition			
Epididymal fat pad weight	g	2.95 ± 0.11	2.04 ± 0.08
Total triglyceride	g	2.66 ± 0.15	1.80 ± 0.10
DNA in fat cells	μg	2.90 ±18	3.70 ±15
Number of fat cells	x 10^7	4.14 ± 0.28[c]	5.29 ± 0.30

[a]Rats were maintained at 5 C for six weeks.

[b]There were 24 rats in each group. Values are the means ± S. E.

[c]7 x 10^{-6} μg DNA per cell.

10^{-6} mg DNA-cell (22) was used to calculate the number of cells in the tissue. The validity of this extrapolation, where polyploidy is not a factor of interference, has been discussed by Vendrely (23). Since triglycerides make up as much as 90% of the total weight of the fat cell, the average triglyceride content of the cells was used as a measure of cell size.

Table II contains the tabulated results obtained from rats maintained either at 25 C or 5 C. Again, as in Table I, the epididymal fat pad weight as well as the total triglyceride content of the fat pads in the group maintained at 5 C are significantly smaller than their controls at 25 C. This decreased pad weight is reflected in the smaller size of the fat cells obtained from the cold-acclimated rats. However, when the total DNA in the tissue originating exclusively from fat cells is determined, the epididymal fat pads from the cold-acclimated rats have a greater amount of DNA and consequently a larger number of fat cells than do rats maintained at 25 C. This would indicate that fat cell proliferation is accelerated in animals exposed to cold. However, it should be noted that the two groups of animals differ in chronological age, the cold-acclimated rats being slightly older than those kept at normal ambient temperature.

In order to determine the effect that age has on fat cell proliferation and growth, we measured cell number and cell size as a function of the rat's growth at the two environmental temperatures. The results are illustrated in Figures 3 and 4. If we consider the rats maintained at normal ambient temperature, we see that the adipocyte content progresses rapidly during the initial growth stage between 100-250 g (Fig. 3). Cell enlargement is also taking place (Fig. 4) but at a rate considerably less than cell proliferation. It should be noted that this is the same weight range in which a linear relationship

TABLE III

Effect of Body Weight and Environmental Temperature on the Norepinephrine Stimulated Lipolysis and Reesterification of Isolated Fat Cells[a]

	Unit of measure	Micromoles fatty acid per μg DNA per 30 min			
		25 C		5 C	
Rat weight	g	268 ± 6[b]	406 ± 6	249 ± 5	406 ± 4
Number of rats		23	12	35	13
Triglyceride-cell	mg x 10^{-5}	4.74 ± 0.28	9.56 ± 0.39	3.12 ± 0.35	5.10 ± 0.10
Lipolysis		570 ± 7[c]	411 ±14	817 ±17	855 ±117
Reesterification		210 ±14[d]	197 ± 8	240 ±27	229 ± 16
		n = 16	n = 15	n = 11	n = 8

[a]Adapted from Hubbard, et al.

[b]Mean ± S. E. of at least three experiments with n replications.

[c]Net glycerol produced x 3 (1-3.5 μg adipocyte DNA per vial).

[d]Net glycerol x 3 produced-net FFA released.

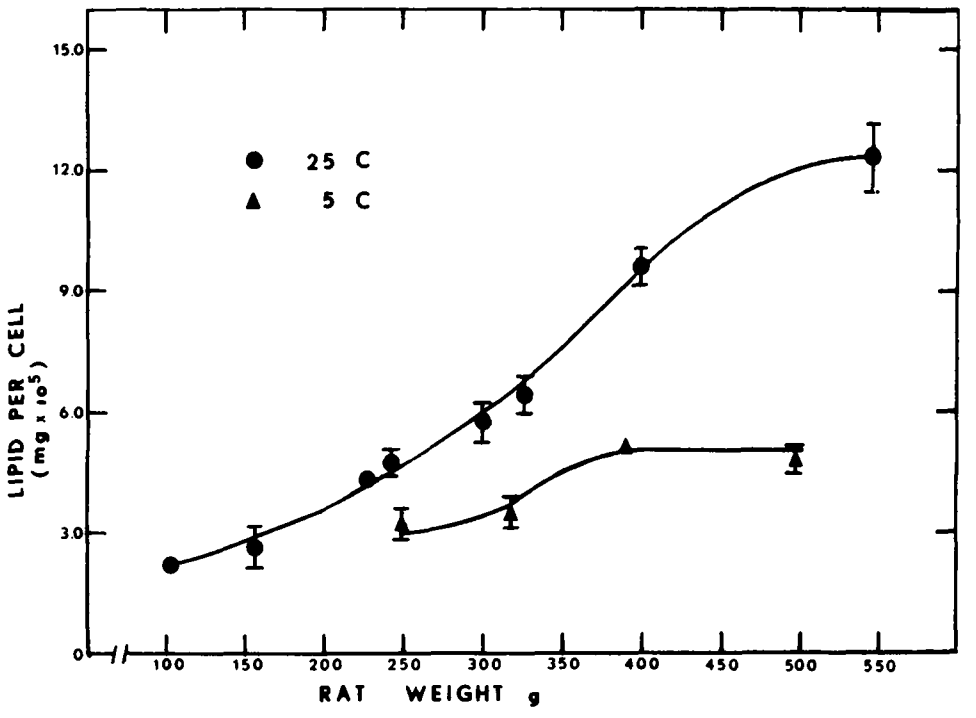

FIG. 4. Relationship between adipocyte size and body weight of the rat. Each point is the mean ± SEM of six separate experiments. All determinations were carried out in triplicate.

between tissue weight and body weight are shown (Fig. 2). As the animal grows larger, cell proliferation is drastically reduced (Fig. 3), whereas cell size increases at an even greater rate (Fig. 4). As seen in Figure 2, it is in this weight range that a disproportionate growth of adipose tissue was noted. It would appear that young growing animals maintained at normal ambient temperature regulate adipose tissue mass primarily by mitotic activity, though some enlargement of preexisting cells does take place. However, in the older mature rat, adipose tissue mass is regulated almost solely by the deposition of lipid within preexisting cells, mitotic activity having almost disappeared. These results do not agree with those reported by Peckham et al. (20) nor with those of Zingg et al. (19). Both reports conclude that the total fat in the fat depot is a function of the number of cells. In fact, Peckham et al. reported that not only was the DNA content of the depot clearly shown to increase with increase in fat deposition, but the reversal of this process, a decrease of DNA content with reduction in fat content, was demonstrated. It should be noted, however, that the total cellularity of the tissue was measured by these authors and not the number of fat cells. Since the major portion of tissue DNA comes from cells other than fat cells (9), it is conceivable that they were measuring changes in the stromal, vascular and fibrous tissue which is present in the intact depot but not in the isolated fat cells.

Examination of Figures 3 and 4 reveals that mitotic activity in the adipose tissue of cold-acclimated rats continues at an accelerated rate throughout the weight range measured. There is little cell enlargement, tissue growth being due primarily to cell proliferation. In animals exposed to cold, there is a definite increase in cell proliferation, resulting in tissue containing a much larger number of cells. On the other hand, the amount of lipid deposited per cell is significantly less in cold-acclimated animals, resulting in adipose tissue which, though it contains more fat cells, is much smaller in mass than that from the control animal maintained at normal ambient temperature.

In most studies with adipose tissue or the isolated fat cells obtained from the tissue, metabolic activity has been expressed per unit weight of the tissue or cells. The number of cells involved in the metabolic reactions being investigated were not taken into account. As is evident from the preceding results, when a comparison is to be made between two groups of animals which have been subjected to different conditions, such a consideration is of prime

importance. If, for example, a comparison were to be made between two rats maintained at normal ambient temperature, but differing widely in body size, 1 g of adipose tissue for the smaller rat would have many more metabolizing cells than 1 g of tissue from the heavier rat. The same may be said in comparing the activity of tissue from cold-exposed rats with that of rats kept at normal ambient temperature. Adipose tissue from a 400 g rat exposed to cold would have more cells than an equivalent amount of tissue from a rat maintained at 25 C. In such studies it is imperative that the activity be expressed in some unit related to cell number rather than to tissue mass. In Table III are tabulated the results from experiments, in which the effect of added norepinephrine on lipolysis and reesterification of isolated fat cells was measured. The rats were kept either at 5 C or 25 C. Animals of different weight within each environmental temperature were studied. In this way it is possible to compare the effect of body weight as well as environmental temperature on these activities. It can be seen that neither rat body weight, fat cell size, nor exposure of the animal to cold affect the norepinephrine-stimulated rate of reesterification. Further examination of the results indicates that adipose tissue from young rats (268 g) maintained at normal ambient temperature is more sensitive to the lipolytic activity of norepinephrine than that from the older rats (406 g). This could be related to the difference in cell size between the two groups. If so, it is a response similar to that reported by Salans et al. (5), who showed that the insulin responsiveness of adipose tissue was dependent upon fat cell size. The larger the fat cells, the less insulin sensitive was the tissue. On the other hand, these results do not agree well with those reported by Benjamin et al. (1), which showed a threefold decrease in esterification and lipolysis in older rats as compared to young ones. Their results, however, did not take into consideration the number of metabolizing cells, but rather, were expressed per unit weight of tissue. It is noteworthy that adipose tissue from cold-acclimated rats is more sensitive to the lipolytic activity of norepinephrine. That this is not due to differences in cell size is evident when we compare lipolysis in the young rats maintained at normal ambient temperature with lipolysis in adipose tissue from rats exposed to cold. The fat cell size in adipose tissue obtained from these animals is not appreciably different, yet lipolysis in the cold-acclimated rats is considerably greater than that in the rats kept at 25 C.

ACKNOWLEDGMENT

Technical assistance by William Matthew, John Ayers and David Dubowick.

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[Received January 9, 1969]

Mass Spectrometry of Long-Chain Aliphatic Aldehydes, Dimethyl Acetals and Alk-1-Enyl Ethers[1]

KIRSTEN CHRISTIANSEN[2], V. MAHADEVAN[3], C. V. VISWANATHAN and R. T. HOLMAN,
The Hormel Institute, University of Minnesota, Austin, Minnesota 55912

ABSTRACT

Mass spectra of long-chain saturated and unsaturated aliphatic aldehydes, dimethyl acetals and alk-1-enyl methyl ethers are reported and discussed. Using deuterium labeled aldehydes, knowledge about the mass spectral fragmentation is obtained. The m/e M-44 ion is formed by a rearrangement different from the mechanism postulated previously for short-chain aldehydes. A series of hydrocarbon fragments with even mass numbers of 68 + 14n (n=0,1,2. . .) appear in the spectra of all three series of compounds.

INTRODUCTION

Aldehydogenic lipids occur widely in nature, and the aldehydes can be released from them by hydrolytic techniques and analyzed as such (1). In the course of analysis of fatty acids, aldehydogenic lipids are subjected to methanolysis, whereby dimethyl acetals of the aldehydes are formed. The dimethyl acetals are converted to the corresponding alk-1-enyl methyl ethers (2,3) during gas chromatography, which leads to some confusion in the analysis of these compounds. The characterization of aldehydes, dimethyl acetals and alk-1-enyl methyl ethers by mass spectrometry was, therefore, undertaken to permit identification of these substances in natural mixtures.

To this end, the mass spectra of dodecanal, tetradecanal, hexadecanal, octadecanal, 9-octadecenal, 9,12-octadecadienal, 9,12,15-octadecatrienal and the corresponding dimethyl acetals and alk-1-enyl methyl ethers were recorded. The fragmentation mechanisms and rearrangements were elucidated by means of deuterium labeling and high resolution mass spectrometry done at the mass spectrometry laboratory of Purdue University using an A.E.I. MS-9/mass spectrometer.

[1]Paper No. 4 in a series, Mass Spectrometry of Lipids. For III, see Arkiv Kemi *29*, 179 (1968).

[2]Permanent address: Institute of Biochemistry C, University of Copenhagen, Radmandsgade 71, 2200 Copenhagen N, Denmark.

[3]Veterans Administration Hospital, Minneapolis, Minn. 55417.

EXPERIMENTAL PROCEDURES

The mass spectra were obtained on a Hitachi Perkin-Elmer RMU-6D instrument. The samples were introduced through the liquid inlet system heated to 125-175 C. The spectra were recorded at high ionization potential (70 or 80 eV) and at the lowest ionization potential which produced a spectrum (nominally 6 to 12 eV). The relative abundance of ions at each mass (m/e) was normalized to the most intense or base peak set at 100.

Preparation of Aldehydes, Dimethyl Acetals and Alk-1-enyl Methyl Ethers

The aldehydes used in this study were prepared by oxidation of the methane sulfonate of the corresponding alcohols with dimethyl sulfoxide as described previously (4). The dimethyl acetals were obtained from the corresponding aldehydes (5). The alk-1-enyl methyl ethers were obtained by thermal loss of methanol from the corresponding dimethyl acetals during preparative gas liquid chromatography (GLC) (3). The purity of the compounds checked by either gas liquid or thin layer chromatography was better than 99%, but both *cis* and *trans* isomers were present. However, mass spectra of the isomers are not distinguishable.

Vic-dideuterated Aldehydes. All members of the series of isomeric *cis* methyl octadecenoates (6) were deuterated using deuterohydrazine as described previously (7). The deuterated compounds were purified by preparative thin layer chromatography on silica gel plates impregnated with $AgNO_3$ using petroleum ether-diethyl ether-acetic acid (90:10:1). The deuterated esters were reduced to the corresponding alcohols with $LiAlH_4$, and were further converted to the aldehydes.

4,4-d_2-Heptadecanal. 1,1-d_2-Tetradecanol was obtained by reduction of methyl tetradecanoate with $LiAlD_4$ (99%, Bio-Rad Laboratories, Richmond, California). The alcohol was converted to the bromide using phosphorus tribromide (8). The Grignard reagent of the bromide was reacted with trimethylene oxide (9) to give the 4,4-d_2-heptadecanol, which was further converted to the aldehyde.

2,2-d_2-Octadecanal. Octadecanal, deuterium oxide and deuterium chloride were shaken at room temperature for 12 hr. The reaction mix-

TABLE I

Relative Intensities[a] of Principal Peaks in the Mass Spectra of Long-Chain Aliphatic Aldehydes

Carbon number, number and position of double bonds:	12:0	14:0	16:0	18:0	18:1ω9	18:2ω6	18:3ω3
Molecular weight:	184	212	240	268	266	264	262
m/e							
M	0.2	0.4	0.2	0.3	2.2	4.0	3.7
M-18	2.6	2.5	1.9	3.1	3.3	---	---
M-28	1.5	1.3	0.4	0.9	---	---	---
M-44	7.3	9.0	1.1	0.9	---	---	---
M-46	3.7	5.6	1.1	0.9	---	---	---
68	26	49	21	21	18	24	13
82	30	75	26	29	18	18	8.4
96	14	44	13	15	12	12	5.1
110	5.5	15	3.6	4.0	3.6	7.0	1.4
124	1.8	8.7	1.6	1.8	1.8	3.0	----
138	3.7	7.2	----	0.9	1.5	2.0	----
152	----	2.3	----	----	1.1	1.0	----
41	100	76	100	84	100	100	100
43	93	98	85	100	58	39	27
57	48	100	40	54	27	11	6.5

[a]Expressed as per cent of base peak.

ture was taken to dryness in vacuo and a fresh portion of deuterium oxide and deuterium chloride was added. The mixture was again shaken for 12 hr and the whole procedure repeated. The2,2-octadecanal was isolated from the reaction mixture by extraction with dry petroleum ether followed by preparative gas liquid chromatography.

5,5-d_2-Octadecanal. 4,4-d_2-Heptadecanol was reacted with methane sulfonyl chloride (10). The mesylate formed was reacted with potassium cyanide to give 4,4-d_2-heptadecanyl cyanide (11) which, after methanolic hydrolysis, gave methyl 5,5-d_2-octadecanoate (11). The aldehyde was obtained by oxidation of the methane sulfonate of the corresponding alcohol, as described above.

TABLE II

Deuterium Content of Some Fragments of Labeled Octadecanals[a]

	Positions of deuterium labeling[b]			
Mol wt	1-d_1	2,3-d_2	4,4-d_2	5,5-d_2
M-18	94	20	72	71
M-19	6	47	25	25
M-20	---	33	3	4
M-44	18	~20	~80	~50
M-45	82	~80	~20	~60

[a]The estimate of the deuterium content of the fragments is rough, as some uncertainty is unavoidable because of small isotopic impurities, unknown isotope effects and, in the case of the M-44 ion, interference from neighboring ions.

[b]Expressed as per cent.

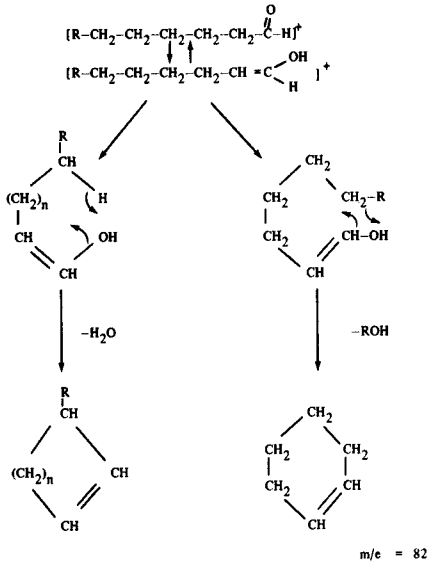
The deuterated aldehydes were purified just before the mass spectral analysis by preparative gas chromatography. The chemical purity was more than 99% and isotopic purity about 90% except for 2,2-d_2-octadecanal (60%).

RESULTS

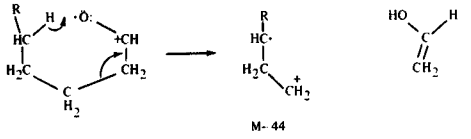
Aldehydes

All the saturated aldehydes gave small but recognizable molecular ion currents. In Figure 1 the mass spectra of tetradecanal at 80 eV and at low voltage are shown. The fragments M-18, M-28 and M-44, which were observed in the mass spectra of short-chain aldehydes (12), were also present in the mass spectra of long-chain aldehydes but with relatively low intensity (Table I). These peaks were, nevertheless, the only peaks in the high mass region of the spectra apart from M-46, which is discussed below. As the chain length increased, the most prominent peak observable in the high mass region of the spectrum was M-18 ($C_{18}H_{34}$ high resolution spectrometry), which presumably was due to loss of water from the molecular ion. In the spectrum of 2,3-d_2-octadecanal, 47% of the M-18 fragment was replaced by a peak at M-19. The spectrum of 1-d_1-hexadecanal showed no M-19 peak, and the spectrum of 2,2-d_2-octadecanal showed that the hydrogen atoms at position 2 are involved. This suggests that the H_2O is split off from an enol form of the aldehyde (Table II). The elimination of water could be via the reactions indicated in Scheme I. In all the spectra of the

isomeric *vic*-dideuterated aldehydes from 3,4-d_2- to 17,18-d_2-, the M-18 is very intense, but measurable amounts of M-19 are present. The intensity of M-19 decreases as the position of the deuterium atoms is moved closer to the end of the hydrocarbon chain. From the spectra of 4,4-d_2-heptadecanal and 5,5-d_2-octadecanal, it is apparent that the hydrogen in positions 4 and 5 contributed 25% each to the elimination (Table II). The elimination of water could be via the reactions indicated in Scheme I:



The formation of the ion M-44 was postulated to involve a β-cleavage and a hydrogen transfer from the γ-position to the oxygen, similar to the McLafferty rearrangement but with a different charge distribution (13). This mechanism cannot explain the formation of the ion M-44, $C_{16}H_{32}$ (high resolution mass spectrometry). The spectrum of 4,4-d_2-heptadecanal showed mainly an M-44 peak; only 20% of this peak is shifted to M-45 and from the spectrum of 5,5-d_2-octadecanal, it is apparent that 60% of the deuterium was lost. The spectra of 1-d_1-hexadecanal and 2,3-d_2-octadecanal both have rather intense M-45 peaks, suggesting that the neutral fragment expelled is C_2H_3DO the same elementary composition as the fragment formed in a McLafferty rearrangement. A suggested mechanism produces a neutral oxygen fragment and a hydrocarbon ion.



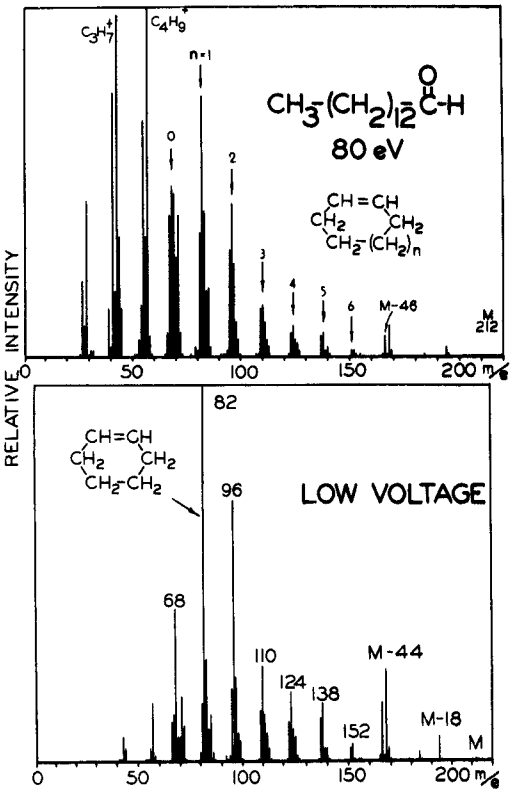


FIG. 1. Mass spectra of tetradecanal at high and low voltages.

According to Gilpin and McLafferty (12), the intensity of the peak decreases with increasing chain length of the aldehyde. Unfortunately, no spectra of deuterated short-chain aldehydes are available to ascertain the transfer of a γ-hydrogen to the oxygen atom as postulated (12). In the long-chain aldehydes, it appears that the hydrogen which rearranged is more from the δ- than from the γ-position.

A peak at M-46 is observed in the spectra of the long-chain saturated aldehydes. The elementary composition of the peak is $C_{16}H_{30}$ determined by high resolution mass spectrometry. The loss of 46 mass units may correspond to loss of 18 + 28 (H_2O + CH_2=CH_2), expulsions frequently observed in other compounds such as alcohols (14).

In the spectra of the saturated aldehydes, hydrocarbon peaks are prominent and are usually the base peaks. Besides the normal series of hydrocarbon fragments, a series of peaks with even mass number was apparent (68 + 14n, where n = 0,1,2 . . .) (Fig. 1). The exact elementary compositions have been determined through high resolution studies of octadecanal, confirming this series to be C_5H_8, C_6H_{10},

TABLE III

Relative Intensities[a] of Ions in the Series 68 + 14n in the Spectra of Some Labeled Aldehydes at Low Voltage

Ion	m/e	Unlabeled	1-d_1	4,4-d_2	5,5-d_2	2,3-d_2	3,4-d_2	4,5-d_2	5,6-d_2	6,7-d_2	7,8-d_2	8,9-d_2
$C_5H_8^+$	68	40	21	35	38	29	23	29	37	51	53	54
	69	5.6	50	52	67	35	26	39	46	14	13	7.8
	70	4.2	11	63	53	17	40	38	15	6.6	4.5	4.7
$C_6H_{10}^+$	82	100	60	48	61	57	31	53	61	69	100	100
	83	27	100	87	100	100	65	100	100	100	69	61
	84	5.9	26	100	98	51	100	96	85	36	27	26
$C_7H_{12}^+$	96	71	52	37	47	45	27	37	42	36	51	57
	97	23	75	70	78	71	47	75	78	63	64	54
	98	6.1	21	70	67	38	71	76	70	46	29	27
$C_8H_{14}^+$	110	24	24	18	21	21	13	18	17	15	15	19
	111	13	31	34	36	27	22	30	30	22	23	22
	112	44	11	26	27	14	27	29	25	18	16	12

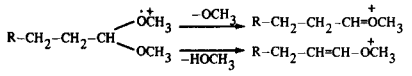
[a]Expressed as per cent of base peak.

C_8H_{12}, etc. From the spectra of a complete series of isomeric *vic*-dideutero aldehydes, it is evident that the carbon atoms involved in this series of fragments are the carbon atoms near the functional group (Table III). For instance, the peak 82 (n=1) is the most prominent peak in the series, is often the base peak in the low voltage spectrum (Fig. 1), and includes carbon atoms 1-6 (Table III). At low ionization voltage this series of peaks became more intense, suggesting that they might arise from a primary decomposition of the molecular ion of low energy requirement. These ions most likely have a ring structure. The spectra of the deuterated compounds show that one of the hydrogens at position 2 is lost (Table III), suggesting a mechanism similar to the one proposed for the formation of m/e M-18, whereby in a 4 center reaction the enol form of the aldehyde in a ring transition state expels ROH (Scheme I). The most intense peaks in the series 68 + 14n are 68, 82 and 96, which may be due to the higher probabilities of forming 5- 6- and 7-membered rings. The spectra of the *gem*-dideutero and the *vic*-dideutero compounds indicate that an exchange of hydrogens from position 2 with hydrogens mainly from positions 4, 5 and 6 has taken place. A similar exchange of hydrogens was also observed in the spectra of deuterated methyl esters. (15).

In contrast to saturated aldehydes, unsaturated aldehydes had molecular ion peaks of measurable intensity (4% in the high voltage spectrum, and 100% in the low voltage spectrum of linoleyl aldehyde) (Table I). The increase in intensity of molecular ions with increasing unsaturation of the molecule has been observed for fatty acid methyl esters (16) and for monoglycerides (17). The mass spectra of unsaturated aldehydes do not exhibit prominent peaks corresponding to the same fragmentations in saturated aldehydes. No peaks at m/e = M-18, M-28, M-44 or M-46 were observed, except for the peak m/e = M-18 in the spectrum of oleyl aldehyde. The base peak in the spectra of the three unsaturated aldehydes was m/e = 41, probably corresponding to the unsaturated hydrocarbon fragment $(CH_2{=}CH\text{-}CH_2)^+$. In the spectra of the unsaturated aldehydes, the low molecular weight portion of the spectrum dominated. In oleyl, linoleyl and linolenyl aldehydes, the series 68 + 14n was also present, but the intensity of these peaks was not as high as in the saturated aldehydes. The intensities of these ions decreased as unsaturation increased, whereas two series of fragments of unsaturated hydrocarbons 66 + 14n and 64 + 14n increased as unsaturation increased.

Dimethyl Acetals

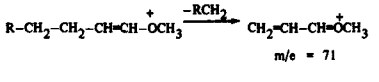
In the spectra of long-chain dimethyl acetals, molecular peaks are not observable, and the peak of highest mass is M-31 (Fig. 2 and Table IV), which can be the loss of OCH_3 from the molecular ion. This fragmentation was observed in the spectra of short-chain homologs (17,18), but is evidently not a favored reaction when the chain length increases. The cleavage which is favored in the long-chain dimethyl acetals involves loss of methanol to give an ion with a vinyl ether structure, M-32.



Loss of 64 mass units from the molecular ion of a long-chain acetal is another fragmentation which has not been reported previously to be a significant mode of fragmentation for short-chain acetals (18,19), but which dominates in longer chain acetals. This fragment originates from the above-mentioned vinyl ether ion through the loss of one more mole of methanol to form an unsaturated hydrocarbon ion of even mass number. The three fragments corresponding to m/e = M-ROH and M-2ROH of dimethyl acetals (R=CH_3) are also characteristic for other acetals, such as 1,1-diethyoxy-, 1,1-dipropoxy-, 1,1-diisopropoxy-, 1,1-dibutoxy- and 1,1-dipentoxy hexadecane. For unsymmetrical acetals, such as 1-methoxy, 1-butoxy hexadecane, M-OCH_3, M-OC_4H_9, M-CH_3OH, M-C_4H_9OH and M- (CH_3OH and C_4H_9OH) were all observed.

In the spectra of short-chain acetals, the peak m/e = 75 $(CH_3O\text{-}CH\text{-}OCH_3)^+$ is usually the base peak, but in the spectra of long-chain acetals this peak is less intense (except dimethoxy dodecane and dimethoxy tetradecane, see Table IV). Moreover, the formation of this ion was found to be influenced by pressure. In the spectrum of 1,1-dimethoxy hexadecane, the intensity of this peak was varied from 99% to 25% when the pressure decreased from 1.3×10^{-5} to 7.0×10^{-7} Torr.

The base peak in all the spectra of the saturated acetals was m/e = 71. This peak can result from the hydrocarbon ion $(C_5H_{11})^+$, or it may have a vinyl ether structure.



The suggestion for the latter possibility is based on the fact that the peak m/e = 71 also is the base peak in the spectra of the alk-1-enyl methyl ethers examined (Fig. 2). Moreover, in the spectra of 1,1-diethoxy

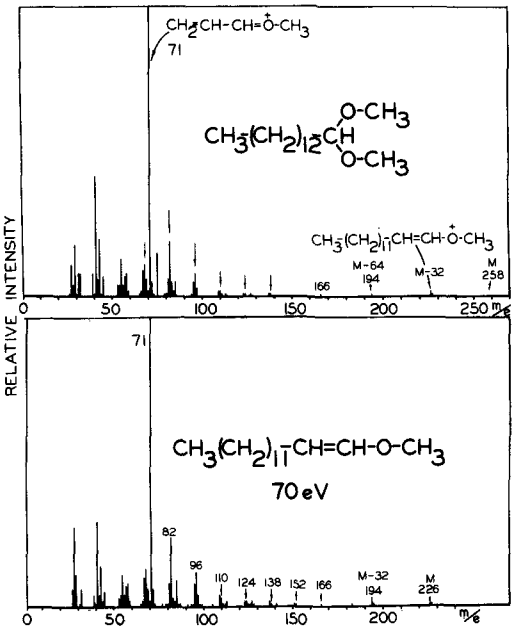
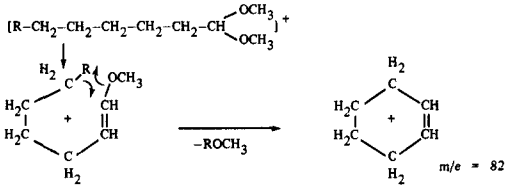


FIG. 2. Mass spectra of 1,1-dimethoxy tetradecane and 1-methoxy-1-tetradecene.

hexadecane, the base peak is not 71 but 85.

As do the spectra of the aldehydes, the spectra of dimethyl acetals showed the series of even-numbered mass peaks, 68 + 14n. Formation of this series of ions could be through a 4 center reaction similar to the postulated mechanism for the formation of the same series of peaks in the spectra of the aldehydes.



Similar to the saturated compounds, the unsaturated acetals also give peaks at m/e = M-31, M-32 and M-64 (Table IV). The peak m/e = 71 is also the base peak in the spectra of unsaturated dimethyl acetals. In the low mass region the spectra were more complex than for saturated compounds. Most of the peaks can be accounted for as saturated and unsaturated hydrocarbon fragments. The intensities of the series 68 + 14n are low and decrease in the order oleyl, linoleyl and linolenyl dimethyl acetals.

The peak m/e = 108 was very intense in the high voltage spectra of linoleyl and linolenyl dimethyl acetals. In the spectrum of methyl

TABLE IV

Relative Intensities[a] of Principal Peaks in the Mass Spectra of Dimethyl Acetals

Carbon number, number and position of double bonds of the aldehyde moiety	12:0	14:0	16:0	18:0	18:1ω9	18:2ω6	18:3ω3
Molecular weight:	230	258	286	314	312	310	308
m/e							
M	---	---	---	---	---	---	---
M-31	1.7	0.8	0.4	0.4	0.7	1.8	1.8
M-32	2.1	2.0	1.6	1.3	2.0	8.0	8.3
M-64	0.9	0.8	1.2	1.3	1.7	0.4	0.5
71	100	100	100	100	85	100	100
75	47	15	3.5	1.6	0.9	---	0.7
68	9.0	11	13	14	15	10	7
82	14	19	22	25	24	7.7	5.4
96	5.7	7.8	9.4	10	18	5.5	4.3
110	2.1	2.0	2.4	2.0	6.6	3.8	2.0
124	0.8	0.8	1.2	1.0	4.1	1.4	0.9
138	1.1	0.8	---	---	4.2	0.8	0.6
152	---	---	---	---	3.6	0.4	0.5

[a]Expressed as per cent of base peak.

linolenate, this peak was interpreted as the hydrocarbon fragment $(C_8H_{12})^+$ (16), corresponding to the member of the series 66 + 14n, in which n = 3. Another series of unsaturated hydrocarbon peaks with mass numbers of 91 + 14n was also observed.

Most of the ions in the spectra of the dimethyl acetals can be explained as fragments derived from the corresponding alk-1-enyl ethers. This may be due to a primary conversion of the acetal to the corresponding vinyl ether by electron impact or by heat. Thermal degradation is indicated by formation of vinyl methyl ethers from dimethyl acetals in a hot gas chromatographic column (2,3), and from 1-alkoxyalkyl acetates (20). Spectra obtained by using the direct inlet system and lower source temperatures still revealed no differences between the spectra of the dimethyl acetals and the 1-alkenyl ethers. That is, the degradation of the dimethyl acetals to the alkenyl ethers could not be avoided under the conditions of the mass spectrometer.

Alk-1-enyl Methyl Ethers

In order to distinguish between the mass spectral fragmentations of dimethyl acetals and their corresponding alk-1-enyl ethers, mass spectra of the latter were also studied. No significant differences were observed between the spectra of these two series of compounds having 16 or 18 carbon atoms (Fig. 2). However, a peak at m/e = 75 is present in the spectra of 1,1-dimethoxy-dodecane and-tetradecane, but is absent in the spectra of the corresponding methyl vinyl ethers. The chain length of these compounds has great influence on the pattern of fragmentation (18), and the observation of Rapport and Norton (21), that the difference in ionization energy between acetals and unsaturated ethers may be diminished for compounds having long hydrocarbon chains, is confirmed by this mass spectral study.

In all three types of compounds (aldehydes, dimethyl acetals and alk-1-enyl ethers), the same series of peaks with even mass number 68 + 14n was observed. This series has not been reported previously to occur in spectra of any other types of lipid derivatives. Ryhage and Stenhagen (22) have reported another even-numbered series, 84 + 14n, in the spectra of dibasic acids. It is, therefore, likely that this series of ions (68 + 14n) is characteristic for the three types of compounds examined here, and that this series can serve to distinguish aldehyde derivatives from methyl esters in mass spectrometric analysis.

ACKNOWLEDGMENTS

Grant numbers HE 03559, HE 02772 and HE 08214, U.S. Public Health Service; methyl 1-d-hexadecanoate, Kwok Kun Sun; prepared aldehydes, acetals and vinyl ethers, F. Phillips; isomeric methyl octadecenoates, F. D. Gunstone; mass spectra, H. W. Hayes. The high resolution studies were done on an A.E.I. MS-9 instrument, courtesy of the Mass Spectrometry Center, Purdue University, Lafayette Indiana. Thanks also to the Hormel Foundation.

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[Received October 11, 1968]

The Lipids of Mitochondria of Human Gray and White Matter

B. GERSTL, L. F. ENG, R. B. HAYMAN and P. BOND,
Department of Pathology, Stanford University School of Medicine,
and Veterans Administration Hospital, Palo Alto, California 94304

ABSTRACT

Mitochondrial enriched fractions were obtained separately from gray and white matter of human and bovine brains. The preparations differed in that choline and ethanolamine glycerophosphatides were present in higher concentration in mitochondria than in gray or white matter from which they were derived. The fatty acid composition also was at variance, palmitic, palmitoleic and linoleic acids being present in considerably higher concentration in the mitochondria of white matter than in the corresponding homogenate, and fatty acids of C_{25} and C_{26} chain length being absent. A deficiency of polyunsaturated fatty acids and the lowest linoleic acid were found in a preparation obtained from a multiple sclerosis brain, a lesser deficiency was noted in the preparation from an emaciated individual.

INTRODUCTION

Lipids are an intrinsic part of the mitochondrial membrane (1) and are essential for the function of these organelles (2-5). Data on mitochondrial lipid composition of rat and guinea pig brain have been reported by several authors (6-12). Corresponding data on mitochondria obtained separately from gray and white matter of brain from either human or experimental animals, however, are wanting. This study was undertaken to obtain such data on mitochondria from brains free of disease as well as from individuals who had died from multiple sclerosis (MS).

MATERIALS AND METHODS

Brain tissue was obtained at autopsy, and white and gray matter were carefully dissected. Age, cause of death, status of nutrition and death-autopsy interval are given in Table I. White matter grossly free of lesions was selected from the two MS brains. For purpose of comparison, mitochondria were prepared also from gray and white matter of two bovine brains kept on ice during transport and processed 2 hr after slaughter.

The tissue was homogenized with 9 vol (v/w) of 0.32 M sucrose and centrifuged at 13,000 x *g* for 45 min; the pellet thus obtained was suspended in 0.88 M sucrose, layered with 0.32 M sucrose, centrifuged at 104,000 x *g* for 60 min, in the cold, and the resulting pellet separated from the interphase which contained the crude myelin. The former, with addition of 0.32 M sucrose was again centrifuged at 900 x *g* for 10 min; the supernatant representing the crude mitochondrial fraction was further purified according to De Robertis et al. (13). Purified myelin was prepared as reported earlier (14). For determination of lipids, extraction was carried out according to Folch et al. (15). The individual lipids were separated by TLC and their amounts determined (16). Recovery of cholesterol and phospholipids ranged between 95-100%, that of galactolipids between 90-100%. Lipid extracts were analyzed for gangliosides by the procedure of Suzuki (17). Quantification of unsubstituted fatty acids was carried out by GLC according to Horning et al. (18). Quantitative control results obtained with National Heart Institute fatty acid standards (A,B,D and F) agreed with the composition

TABLE I

Age, Diagnosis, Nutritional Status and Death-Autopsy Interval of Brain Specimens

Specimen	Age	Sex	Diagnosis	Status of nutrition	Interval-death to autopsy
67-13	45	M	Myocardial infarct, severe arteriosclerosis	Emaciated	13 hr
6F-365	41	M	Generalized arteriosclerosis; left adrenal hyperplasia	Well nourished	6 hr
6F-206	26	M	Carcinoma of testes	Normal. Well nourished	a
68-98	56	M	Carcinoma of lung	Well nourished	3 hr
St. (MS)	63	M	Multiple sclerosis	Well nourished	5 hr
NH. (MS)	43	F	Multiple sclerosis	Undernourished	a

[a]Unknown

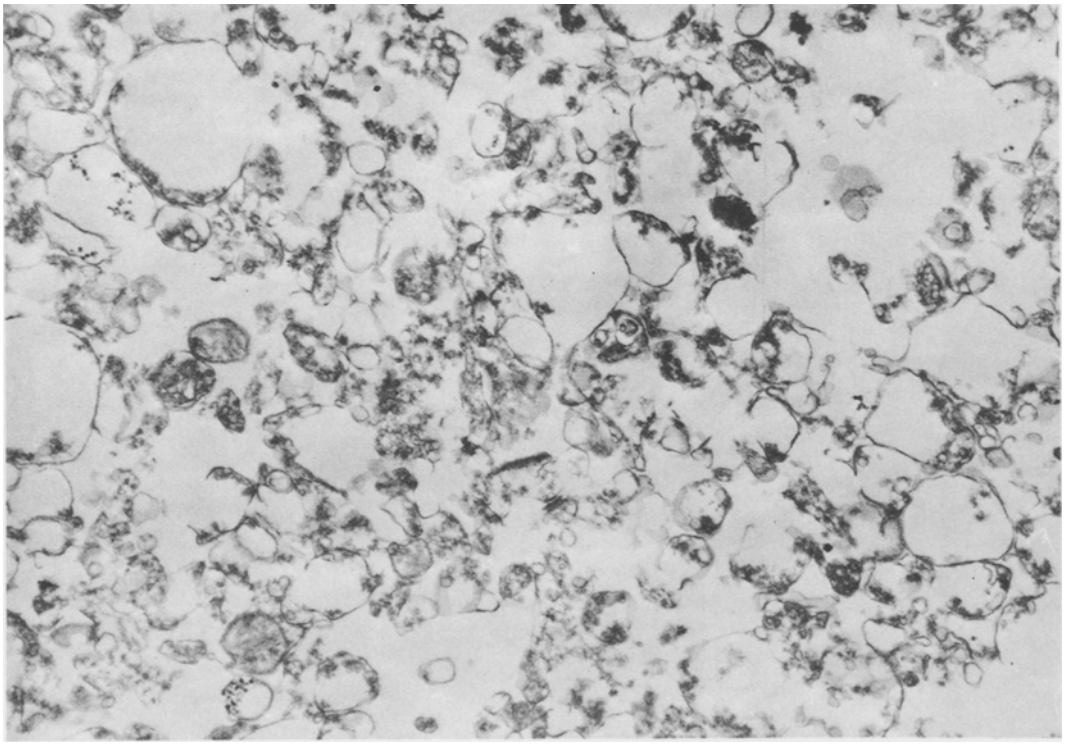

FIG. 1. Electron micrograph of human gray matter mitochondria. Thin sections stained with uranyl acetate and lead citrate. (X 23,000, reduced approximately 17%)

given with a relative error of less than 3% for major, and of less than 5% for minor components. All analyses were done in duplicate except those on the sulfatides of mitochondrial preparations. The TLC procedure did not resolve the cardiolipin completely from the EGP fraction when applied to the human material, therefore, these fractions were pooled. In the case of bovine brain mitochondrial preparations, cardiolipin was completely separated and amounted to about 4% of the total phospholipids. In order to study the fatty acid composition of the galactolipids of mitochondria, mitochondrial-enriched fractions from larger

TABLE II

Lipid Composition of Mitochondria and of Corresponding Whole Gray Matter (in mole % of total lipids)[a]

Specimen	Cholesterol	Lipid phosphorus	SGP+IGP	CGP	EGP	Total plasmalogens	Sphingomyelin	Galactolipids	SDH[b]
Mitochondria									
67-13	33.5	63.3	7.1	25.1	24.9	c	6.2	3.2	0.79
6F-365	31.8	64.9	6.9	26.3	23.9	c	5.6	3.2	c
St. (MS)	33.5	61.0	8.6	24.6	22.4	10.1	5.4	5.5	1.60
Bovine	30.9	65.8	4.3	27.0	27.0	10.9	7.5	3.3	1.61
Gray matter, homogenate									
67-13	36.4	57.8	8.8	21.4	19.4	9.9	8.3	5.8	0.33
St. (MS)	37.5	56.8	10.5	22.7	18.9	10.4	4.7	5.6	c
NH. (MS)	35.8	57.8	8.2	22.4	20.3	9.7	6.9	6.4	c
Bovine	34.9	58.3	9.4	21.9	18.0	9.9	9.0	6.8	0.71

[a]Total lipids, 100%.

[b]Moles of dye reduced per 1 hr/kg protein.

[c]Not done.

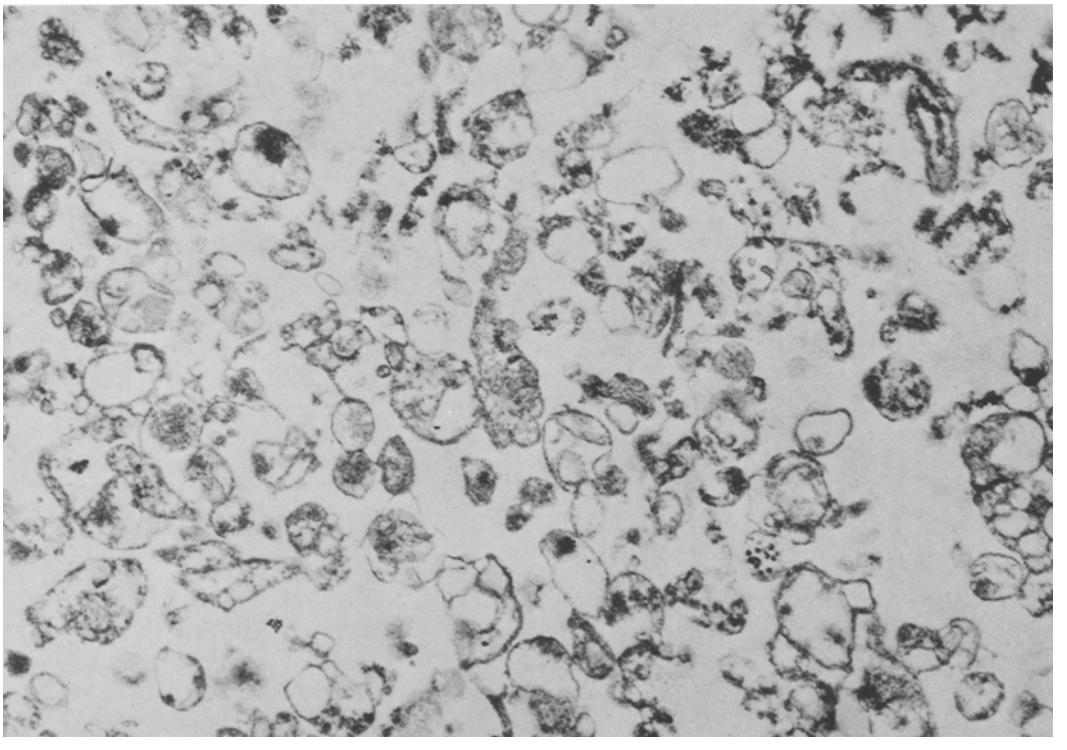

FIG. 2. Electron micrograph of human white matter mitochondria. Thin sections stained with uranyl acetate and lead citrate. (X 23,000, reduced approximately 17%)

amounts of tissue of an additional brain (68-98) were prepared as described. Amounts were obtained from white matter sufficient to allow separation of sulfatides and cerebrosides by two-dimensional TLC (16). The amount of galactolipids recovered from gray matter mitochondria was insufficient for this and, therefore, the combined cerebroside-sulfatide (galactolipid) fraction was processed. The unsubstituted and 2-hydroxy fatty acids were separated and quantified by GLC as reported earlier (19).

For estimation of succinic dehydrogenase activity (SDH) the tetrazolium dye reduction method of Glick and Nayyar (20), as modified by Laatsch et al. (21), was used and phosphatase activity was estimated by using *p*-nitro phenyl phosphate as substrate (Sigma substrate 104 colorimetric, Sigma Chemical Company, St. Louis, Mo). Protein content was determined according to Lowry et al. (22).

Histologic sections prepared on aliquots fixed with formalin, embedded in paraffin and stained with H & E, phosphotungstic acid, or Luxol blue, were screened for admixture of other tissue, particularly, myelin. In three instances, mitochondrial-enriched fractions were fixed in buffered osmium tetroxide, embedded in Araldite, and ultrathin sections were prepared therefrom for electron microscopy (EM) study.

RESULTS

Morphology of the mitochondria (Fig. 1,2) was similar to that illustrated by Lǿvtrup (7) on rat preparations. The heterogeneity of appearance may be, in addition to post-mortem changes, due to different derivation of the mitochondria. Myelin admixture as seen in the light and EM preparations was minimal in mitochondrial fractions of gray matter and was estimated at less than 10% of total material in those of white matter. The amount of mitochondria recovered from the brain as estimated by protein determination was, on an average, 9% for gray and 1% for white matter. These data reflect greater cellularities of the former, and compare to 5.26% of total protein for mitochondrial preparations from whole guinea pig brains (23).

Comparison of the lipid composition of the mitochondrial-enriched fraction with that of gray matter from which it was obtained (Table II) revealed that the concentration of cholesterol in the former was lower, and that of total

TABLE III

Lipid Composition of Mitochondria and of Corresponding Whole White Matter (in mole % of total lipids)[a]

Specimen	Cholesterol	Lipid phos-phorus	SGP+ IGP	CGP	EGP	Total plasma-logens	Sphingo-myelin	Galacto-lipids	SDH[b]
Mitochondria									
67-13	40.7	47.8	7.2	15.6	18.5	c	6.5	11.5	0.36
6F-206	41.1	43.3	7.8	15.5	13.4	c	6.6	15.6	c
6F-365	44.3	47.2	9.4	15.0	16.0	c	6.8	8.5	0.34
St. (MS)	37.2	42.7	7.4	14.6	15.0	8.7	5.7	20.1	1.70
NH. (MS)	36.6	48.8	8.0	16.8	16.8	c	7.1	14.6	c
Bovine	37.5	59.5	6.5	21.2	21.7	c	10.1	3.0	2.16
White Matter, Homogenate Controls[d]									
N=7	41.7	37.5	9.1	9.4	13.7	11.5	5.3	20.1	0.04
St. (MS)	39.2	41.7	8.9	11.3	13.9	13.7	7.6	19.2	c
NH. (MS)	43.1	40.3	9.5	9.3	13.8	13.7	7.7	16.5	c
Bovine	44.0	40.4	9.6	9.4	13.1	13.1	8.3	15.6	0.09

[a]Total lipids, 100%.
[b]Moles of dye reduced per 1 hr/kg protein.
[c]Not done.
[d]N, number of specimens analyzed.

phospholipids, choline glycerophosphatide (CGP) and ethanolamine glycerophosphatide (EGP) higher, than in whole gray matter. Plasmalogens were present in a concentration similar to, and sphingolipids lower than in gray matter. Proteins made up approximately 60% of the total dry weight. SDH activity in the preparation of the MS brain was as high as in that of the bovine brain, while it was low in case 67-13 where the death-autopsy interval was 13 hr. The high SDH activity in the preparation of the MS brain indicated good preservation of material. Acid phosphatase activity was 1.69 units/mg protein for gray matter, 0.18 for its mitochondria, (68-98), while corresponding figures for white matter were 1.55 and 1.36, respectively. There was no difference in lipid distribution between the mitochondria from normal and MS gray matter. Gangliosides were absent.

The concentration of cholesterol in the mitochondrial-enriched fraction of normal white matter was similar to, and that of total phospholipids, EGP and CGP, higher than in the homogenate of white matter (Table III). The data on whole white matter correspond to those reported earlier (14). The bovine mitochondria showed higher proportions of total phospholipids, CGP and EGP, than the human material. The concentration of galactolipids was about one half of that in white matter, while that of sphingomyelin showed no difference between the two materials. Gangliosides were absent.

TABLE IV

Unsubstituted Fatty Acids of Mitochondria and of Corresponding Whole Gray Matter (in mole %)

	Methyl ester							
Specimen	16:0	16:1	18:0	20:0 22:0 24:0	18:1	20:1 22:1 24:1	18:2	20:4 22:4 22:6
Mitochondria								
67-13	27.9	2.8	23.9	0.4	18.9	0.4	0.8	20.8
6F-365	27.3	2.5	24.6	0.4	18.3	0.8	1.6	20.0
St. (MS)	29.1	2.6	24.8	0.4	18.5	0.4	1.5	19.4
Bovine	29.7	3.1	24.2	a	18.1	0.3	1.2	19.3
Gray Matter, homogenate								
67-13	27.1	2.3	26.9	0.3	18.1	0.6	0.9	18.8
6F-206	28.6	2.0	24.4	0.4	20.2	1.7	0.9	17.1
St. (MS)	30.5	1.9	24.0	0.5	18.1	1.3	0.7	19.6
Bovine	30.6	2.1	25.4	0.3	20.8	0.6	0.6	15.9

[a]Not found.

TABLE V

Unsubstituted Fatty Acids of Mitochondria and of Corresponding Whole White Matter (in mole %)

	Methyl ester									
Specimen	16:0	16:1	18:0	20:0 22:0 24:0	18:1	20:1 22:1 24:1	18:2	20:4 22:4 22:6	25:0 26:0	25:1 26:1
Mitochondria										
67-13	20.9	2.8	18.5	1.8	32.8	4.1	0.9	11.1	a	a
6F-206	19.6	2.4	21.1	1.4	29.5	3.1	1.8	15.3	a	a
NH. (MS)	15.8	1.5	21.9	2.8	32.5	6.8	0.6	11.9	a	a
St. (MS)	23.7	2.0	21.2	1.7	27.0	3.4	1.1	7.9	a	a
Bovine	28.5	5.2	15.6	2.1	25.5	3.5	1.0	7.9	a	a
White Matter, homogenate										
67-13	13.7	1.8	20.5	2.8	36.1	8.5	0.3	8.2	0.8	2.1
6F-206	12.9	1.2	21.9	2.6	36.7	9.1	0.4	7.3	1.1	2.9
NH. (MS)	14.9	1.2	21.1	1.9	38.5	8.2	0.3	7.4	0.6	2.0
St. (MS)	14.9	1.0	21.5	1.6	38.8	6.9	0.3	6.7	0.5	2.4
Bovine	14.5	1.5	17.8	4.3	37.7	9.6	0.2	6.7	0.8	2.2

[a]Not found.

The distribution of regular saturated and monounsaturated fatty acids in the mitochondria of gray matter (Table IV) was similar to that in whole gray matter. Polyunsaturated fatty acids (PUFA) were found in slightly higher proportions in three of the four mitochondrial preparations than in gray matter. This was not the case in the MS sample.

The distribution of fatty acids in the mitochondrial-fraction of human white matter (Table V) differed in several respects from that in the total homogenate. The proportion of palmitic, palmitoleic and linoleic acids was considerably higher in the mitochondrial-enriched fraction, while that of the long chain monounsaturated acids was lower than in whole white matter; fatty acids of C_{25} and C_{26} chain length were absent. When a total of 10 μmoles of fatty acid methylates of gray matter mitochondria were applied to TLC, hydroxy fatty acids were not discernible. In the mitochondrial extracts of white matter, traces of hydroxy fatty acids were present on TLC but insufficient in amount for further analysis.

In the specimen 68-98, the unsubstituted fatty acids (Table VI) of the homogenate of white matter cerebrosides and sulfatides showed a distribution similar to that reported by O'Brien and Sampson (24) and by Svennerholm and Stallberg-Stenhagen (25), except for a greater percentage of $C_{18:1}$ in both the cerebrosides and sulfatides. The corresponding mitochondrial extracts differed by greater percentages of $C_{20:0}$, $C_{22:0}$, $C_{24:0}$ and $C_{26:0}$, and substantially less $C_{24:1}$ acids.

The distribution of the hydroxy fatty acids

TABLE VI

Unsubstituted Fatty Acid Composition (in mole %)

Fatty acid	Cerebrosides of white matter		Sulfatides of white matter		Galactolipids of white matter	
	Homogenate	Mitochondria	Homogenate	Mitochondria	Homogenate	Mitochondria
16:0	2.0	2.4	8.9	8.4	3.8	3.7
18:0	12.2	11.5	6.5	7.7	6.3	6.1
18:1	4.2	4.7	4.1	7.1	5.3	5.6
20:0	1.2	6.2	2.3	6.8	2.1	5.5
22:0	2.4	8.5	2.6	7.9	3.5	7.3
22:1	1.0	Trace	1.1	Trace	Trace	5.4
23:0	4.7	5.7	3.9	5.7	3.6	5.0
24:0	11.9	19.2	17.1	21.4	8.0	13.0
24:1	39.6	14.9	36.9	7.3	41.8	21.2
25:0	4.0	5.4	4.6	9.1	3.6	5.9
25:1	8.6	5.6	8.4	4.1	11.6	6.1
26:0	1.5	7.6	2.3	6.7	2.0	7.3
26:1	6.7	8.3	7.3	7.8	8.4	7.9

TABLE VII

Hydroxy Fatty Acid Composition (in mole %)

Fatty acid	Cerebrosides of white matter			Sulfatides of white matter		Galactolipids of white matter	
	Homogenate	Mitochondria a	Mitochondria b	Homogenate	Mitochondria	Homogenate	Mitochondria
18:0	8.3	24.0	22.0	8.9	18.6	1.0	8.8
20:0	1.6	6.5	7.5	2.3	17.6	0.5	6.2
22:0	7.3	14.1	17.1	7.1	14.7	10.1	19.6
23:0	15.9	19.6	20.4	14.9	17.3	19.2	23.8
24:0	37.4	22.8	25.2	30.3	19.8	29.9	28.7
24:1	17.1	13.0	7.8	11.4	12.0	26.6	12.9
25:0	5.9	Trace	Trace	3.8	Trace	4.6	c
25:1	2.9	Trace	Trace	7.5	Trace	3.6	c
26:0	1.4	c	Trace	6.9	Trace	1.0	c
26:1	2.3	c	Trace	7.0	Trace	3.5	c

[c]Not done.

(Table VII) of the cerebrosides of the white matter homogenate again was similar to that reported by Svennerholm save for a greater percentage of $C_{18:0}$, while that of mitochondrial cerebrosides had greater amounts of $C_{18:0}$, $C_{20:0}$, $C_{22:0}$ and less of $C_{24:1}$. Hydroxy fatty acids of C_{25} and C_{26} chain length were practically absent. Similar observations were noted for the corresponding sulfatides of white matter.

The galactolipids of gray matter yielded hydroxy fatty acids almost identical in distribution to that calculated from Svennerholm's data while those of the corresponding mitochondria revealed greater percentages of $C_{18:0}$ and $C_{20:0}$ and absence of the longer chain fatty acids.

The galactolipid fraction of mitochondria of gray matter was insufficient in amount to allow separation of cerebrosides and sulfatides. Thus, the fatty acids of the combined mitochondrial galactolipids were separated into unsubstituted and hydroxy acids. The distribution of the unsubstituted acids of the homogenate resembled that given by O'brien and by Svennerholm, when their data are calculated for the combined fractions, assuming a ratio of cerebroside-sulfatide of 7:3, and one of unsubstituted-hydroxy fatty acids in the cerebrosides of 5:5 and in the sulfatides of 7.5:2.5. The corresponding mitochondrial extract revealed higher proportions of $C_{20:0}$, $C_{22:0}$ and $C_{26:0}$, and lesser ones of $C_{24:1}$.

DISCUSSION

The results presented reveal that mitochondria derived separately from gray and white matter differ significantly in their lipid composition.

It is noteworthy that the mitochondrial preparations of gray matter like those obtained from whole brain studied by other authors (6-12,26) are of heterogeneous origin, namely, neurons, axons, as well as glia cells, while the corresponding preparations of white matter derive primarily from glia cells. The data on cholesterol and total phospholipids of gray matter mitochondria correspond to those of Biran and Bartley (6) and of Seminario et al. (9). The data on EGP of these two authors are slightly higher than, and those on plasmalogens by Biran and Bartley (6) almost identical to ours on gray matter mitochondria. These data, together with a fatty acid distribution similar to that given by Witting et al. (27) for whole rat brain mitochondria, suggest that most of the mitochondria in the preparations of these authors were derived from gray matter. Lϕvtrup (7), Smith and Eng (26) and Cuzner et al. (12), however, reported lower cholesterol and higher phospholipid, and Parsons and Basford (28) reported higher sphingomyelin concentration of animal brain mitochondria than found in mitochondria of either gray or white matter of human brains. The differences are possibly due to the admixture of other subcellular fragments. The absence of gangliosides suggests that synaptosomes were not part of the contaminant. The acid phosphatase activity in both the gray and white matter preparation suggests either presence of lysosomes or their rupture, with consequent release of this hydrolase as a post-mortem event. Since mitochondria and myelin undergo post-mortem (anoxic) changes, the degree of purity and intactness of preparations as secured from experimental animals could not be expected. A relatively high content of cholesterol seems to be characteristic for brain mitochondria which distinguishes them from mitochondria of liver and kidney (7). Radin et al. (29) reported that the fatty acid composition of the microsomal phospholipids derived from rat brain gray matter dif-

fered from that of white matter.

Phospholipids are present in higher concentration in mitochondria than in whole gray matter. They consist mostly of nearly equal amounts of CGP and EGP, while in guinea pig preparations the corresponding proportions were 25.6% and 18.9%, respectively (8). A preponderance of CGP over EGP has been noted also for mitochondria of liver (30).

The fatty acid distribution in mitochondria of white matter differs from that in whole white matter or myelin, a multilayered biomembrane. The sum of long chain monounsaturated fatty acids in the mitochondrial fraction is only about one half of that in myelin, while the reverse is true for the tetra-, penta- and hexaenoic acids. Fatty acids of C_{25} and C_{26} chain length are not measurable. In their isolated and concentrated galactolipid fractions, however, unsubstituted but not hydroxy fatty acids of this chain length could be demonstrated. The greater proportion of medium chain length fatty acids raises the question whether the galactolipids containing esterified fatty acids which are almost entirely of medium chain length (31) are predominantly present in the subcellular fraction. If the cerebrosides found in the mitochondrial fraction were due entirely to admixture of myelin, all the long chain fatty acids should be represented. Another possibility would be post-mortem breakdown of myelin with release of some fragments contaminating the mitochondrial fraction.

A deficit of PUFA has been suggested as one factor in the chemical pathology of MS (32) and, indeed, one of linoleic and arachidonic acid has been reported for the lecithin fraction of white matter of MS brains (33). In the experiments reported here, the lowest linoleic acid value is noted in the mitochondria of one MS white matter (NH) associated with a deficit of other PUFA. A lesser deficit was noted in the preparation of an emaciated individual (67-13). Whether these differences may be attributed to nutritional factors (10), or other factors, remains to be established by further studies.

ACKNOWLEDGMENTS

Electron micrography by R. E. Smith. This work was supported by the U.S. Veterans Administration and by U.S.P.H.S. Grant NB-03821.

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[Received April 3, 1968]

Glyceryl Ether, Wax Ester and Triglyceride Composition of the Mouse Preputial Gland[1,2]

GAIL SANSONE[3] and JAMES G. HAMILTON[4], Department of Biochemistry, Tulane University School of Medicine, New Orleans, Louisiana 70112

ABSTRACT

Major lipid classes of the preputial gland of the mouse have been identified as wax ester, neutral plasmalogen, glyceryl ether diester and triglyceride. The chain lengths and degree of unsaturation in the aliphatic moieties of the alk-1-enyl and alkyl glyceryl ethers are similar to those of the fatty alcohols of the wax ester fraction. This lends support to the theory that long chain fatty alcohols can be direct precursors of the aliphatic chains of glyceryl ethers. The striking qualitative, as well as quantitative, similarities between the alkyl and alk-1-enyl moieties of the glyceryl ethers in the neutral lipid fraction suggest that they share a common pathway of biosynthesis or are interconvertible. Neutral plasmalogens and glyceryl ether diesters contain significant amounts of odd-numbered and branched fatty acids, unlike the fatty acids of the triglycerides; therefore, the biosynthesis of neutral plasmalogens and glyceryl ether diesters may not be related to the biosynthesis of triglycerides.

INTRODUCTION

The preputial gland of the rat has been used by many investigators as a model sebaceous gland to study the effects of steroid hormones on lipid metabolism (1). However, the preputial gland of the mouse has received very little attention.

Thin layer chromatography (TLC) of the mouse preputial gland lipid extract showed the presence of large quantities of unknown lipid. In a preliminary report (2), the unknown lipid classes were identified as neutral plasmalogen, glyceryl ether diesters and wax esters. A more detailed study of the metabolism of the mouse preputial gland has also been reported (3).

Recent investigations have implicated long chain fatty alcohols as immediate precursors of the alkyl glyceryl ethers (4,5). The preputial gland of the mouse contains a unique combination of wax esters and glyceryl ethers. This investigation was undertaken to characterize the neutral lipids of the mouse preputial gland and to study the structural and metabolic relationships of the alcohols in the wax ester fractions to the alkyl and alk-1-enyl moieties of the glyceryl ethers.

MATERIALS AND METHODS

Materials

Solvents were reagent grade or otherwise distilled. Wax ester standards were synthesized from fatty acids and fatty alcohols in the presence of oxalyl chloride (6). Synthetic glyceryl ether diester standards were purchased from Analabs, Inc., Hamden, Conn. Neutral plasmalogen, isolated from ratfish liver oil, was a gift from Helmut Mangold of the Hormel Institute. Its physical and chemical properties have been described (7). Fatty acid methyl ester standards for gas chromatography were purchased from Applied Science Laboratories, State College, Pa. Alcohol acetate standards were synthesized from lauryl, tetradecyl, cetyl and octadecyl alcohols (8).

Animals

Adult male rats (Sprague-Dawley strain) and mice (ICR strain), obtained from Charles River Breeding Laboratories, Wilmington, Del., were individually caged and fed a diet of Purina Lab Chow ad lib. For the identification and quantitative studies, 10 g of lipid was obtained from the excised preputial glands of 100 three month old male mice.

Methods

Total lipids were extracted from the preputial glands as described by Folch et al. (9). Phospholipids were separated from neutral lipids by adsorption on silicic acid (10). The neutral lipids were eluted with diethyl ether.

Wax esters were saponified with 9 ml 10% KOH in ethanol-water (9:1 v/v) under reflux in a nitrogen atmosphere for 2 to 4 hr. Saponification of the neutral plasmalogen and glyceryl ether diester fractions was accomplished by refluxing in 0.5 N ethanolic KOH in a nitrogen

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[1]Presented at the AOCS Meeting, Chicago, October, 1967.

[2]Taken from a dissertation submitted to Tulane University in partial fulfillment of the requirement for the degree of Doctor of Philosophy by Gail Sansone.

[3]Present Address: Division of Dermatology, Harbor General Hospital, Torrance, Calif.

[4]Present Address: Division of Biochemical Nutrition, Hoffmann LaRoche Inc., Nutley, N.J.

atmosphere for 1 hr. The nonsaponifiable fraction was extracted 5-10 times with equal volumes of hexane.

For GLC analysis, trimethylsilyl ether (TMS) derivatives were made of the glycerol alkyl ethers (11). The fatty acids obtained from the glyceryl ether diesters, neutral plasmalogens and wax esters were esterified with BF_3 in methanol (12). Aldehydes were liberated from the alk-1-enyl glyceryl ethers by HCl and reduced to alcohols by the method of Katz and Keeney (13). The alcohols were converted to acetates for GLC (8).

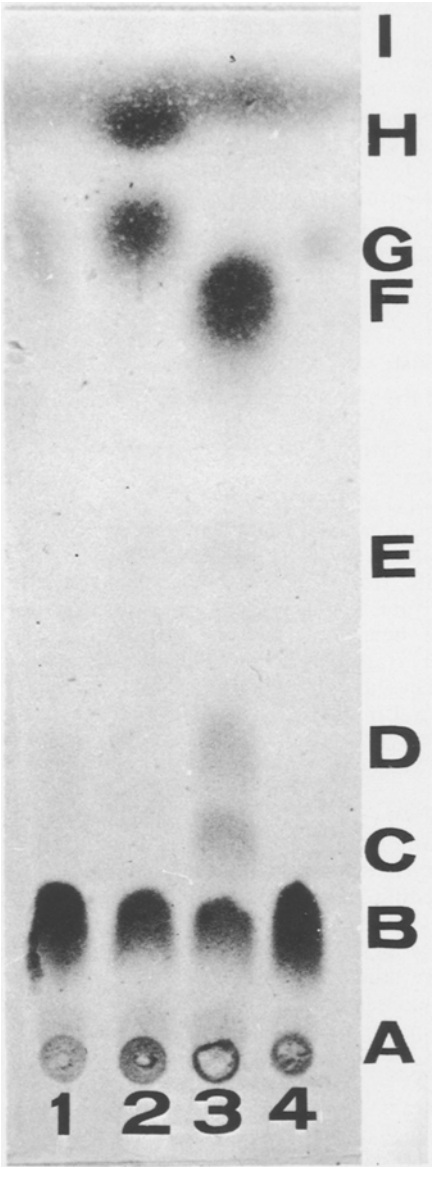

FIG. 1. TLC of lipids of rat and mouse preputial glands. (1) castrated male rat; (2) normal male rat; (3) normal male mouse; (4) corn oil triglyceride. The letters at the right side of the chromatogram are to identify the following: (A) origin, (B) triglyceride, (C) glyceryl ether diester, (D) neutral plasmalogen, (E) unidentified lipid, (F) wax ester, (G) sterol ester, (H) squalene and (I) solvent front. Solvent system used was hexane-diethyl ether (95:5 v/v).

Preparative Chromatography

Preputial gland neutral lipids were fractionated by column chromatography on Mallinckrodt silicic acid, 100 mesh. Columns were prepared by pouring a silicic acid slurry in hexane into a column (25 x 2.5 cm). The lipids were put onto the column in hexane, and the various fractions, wax esters, neutral plasmalogens, glyceryl ether diesters and triglycerides were eluted with mixtures of benzene and hexane (Table I). Each fraction was identified by glass fiber paper chromatography in a solvent system of isooctane-isopropyl acetate (100:1.5 v/v).

Neutral plasmalogens, glyceryl ether diesters and triglycerides isolated by column chromatography were further purified by TLC on Silica Gel G in a solvent system of hexane-diethyl ether (95:5 v/v).

Glass Fiber Paper Chromatography

Glass fiber paper (GFP) type ITLC-SG, impregnated with silica gel, was used to identify all lipids. The chromatographic procedures for GFP have been published in detail (14). Wax ester, neutral plasmalogen, glyceryl ether diesters and triglycerides were separated on GFP in a solvent system of isooctane-isopropyl acetate (100:2 v/v) or isooctane-chloroform

TABLE I

Elution Sequence of Preputial Gland Neutral Lipids From a Silicic Acid Column[a]

Eluent		Volume of eluent	Eluent contained
Hexane	Benzene v/v		
100	---	150 ml	Hydrocarbon
100	5	100 ml	Wax ester
100	10	100 ml	Wax ester
100	20	100 ml	Wax ester
100	30	100 ml	Wax ester
100	50	150 ml	Neutral plasmalogen
100	75	200 ml	NP + GEDE[b]
100	100	200 ml	GEDE
100	150	300 ml	GEDE + triglyceride
---	200	400 ml	Triglyceride

[a]Column dimensions, 25 x 2.5 cm.

[b]NP, neutral plasmalogen; GEDE, glyceryl ether diester.

(100:20 v/v). Alk-1-enyl and alkyl ethers of glycerol were separated in isooctane-chloroform (1:1 v/v).

Gas Liquid Chromatography

A Barber-Colman gas liquid chromatograph (GLC) equipped with an argon ionization detector was used. TMS derivatives of alkyl ethers were analyzed on a 6 ft x 4 mm I.D. u-shaped column packed with 3% SE-30 on 100-110 mesh ABS operating at 210 C, detector temperature 220 C, and argon carrier gas flow rate 45 ml/min. Fatty acid methyl esters were analyzed on a 6 ft x 4 mm I.D. u-shaped column packed with 14% EGSS-X 100-200 mesh on Gas Chrom P, operating at 125 C with a flow rate of 30 ml/min. Fatty alcohol acetates were analyzed on the EGSS-X column at 150 C.

Infrared Spectroscopy

The purified fractions of glyceryl ether diesters and neutral plasmalogens were analyzed as thin films between NaCl plates or in KBr pellets containing 0.1% sample on a Perkin-Elmer Model 237 spectrophotometer.

RESULTS AND DISCUSSION

The lipid fractions of the mouse preputial gland were identified as wax esters (WE), neutral plasmalogens (NP), glyceryl ether diesters (GEDE) and triglycerides (TG) by adsorption chromatography, GLC and infrared spectroscopy (IR).

The differences between the lipid classes of rat and mouse preputial glands are shown in Figure 1. The neutral lipid fraction of the mouse preputial gland extract contains high levels of NP and GEDE (Fig. 1, Lane 3). These lipids are not detectable by TLC in the rat preputial gland (Fig. 1, Lane 2). The mouse gland also contains other unidentified lipids. One, which is visible at the chromatographic load used, appears in Figure 1, Lane 3, Area E.

The GEDE and NP isolated from the preputial gland by column chromatography (Table 1) and preparative TLC are shown in Figure 2, along with standards. The R_f values of the isolated glyceryl ethers are similar to the standards. The isolated NP and GEDE were subjected to alkaline hydrolysis followed by chromatography of the nonsaponifiable fractions. The NP fraction yielded alk-1-enyl glyceryl ether upon hydrolysis, and the GEDE fraction yielded alkyl glyceryl ether. The identity of these compounds was verified by TLC and GLC with appropriate standards.

The IR spectra of the NP and GEDE isolated from the preputial gland are compared to synthetic GEDE in Figure 3. Characteristic absorptions for ether (8.95μ) and carbonyl

TABLE II

Per Cent Composition of Glycerol Alk-1-enyl Ethers, Glycerol Alkyl Ethers and Alcohols From Wax Esters in the Preputial Gland of the Mouse

Chain length and no. of double bonds[a]	Glycerol alk-1-enyl ethers[b]	Glycerol alkyl ether[c]	Wax ester alcohols[d]
14:0	23.9[e]	21.6	4.1
14:1	12.0	13.2	8.6
15:0	<1	<1	<1
16:0	49.6	50.5	79.1
16:1	13.5	11.9	5.4
18:0	0.8	1.9	2.3

[a]Chain length and degree of unsaturation determined from log plot and co-chromatography with standards.

[b]Analyzed as alcohol acetates derived from the alk-1-enyl ethers.

[c]Analyzed as trimethyl silyl ether derivatives.

[d]Analyzed as acetates.

[e]Quantitation by determination of peak areas by triangulation.

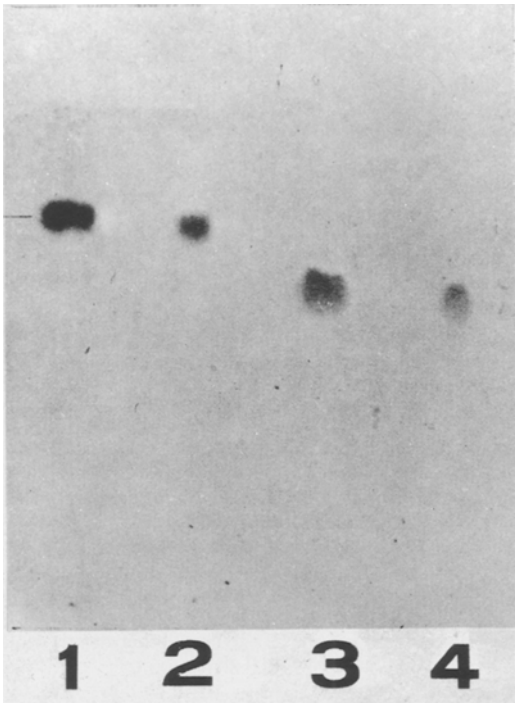

FIG. 2. Chromatogram of standard neutral plasmalogen (1), neutral plasmalogen isolated from the mouse preputial gland (2), standard glyceryl ether diester (3) and glyceryl ether diester from preputial gland (4). Solvent system used was hexane-chloroform (100:20 v/v).

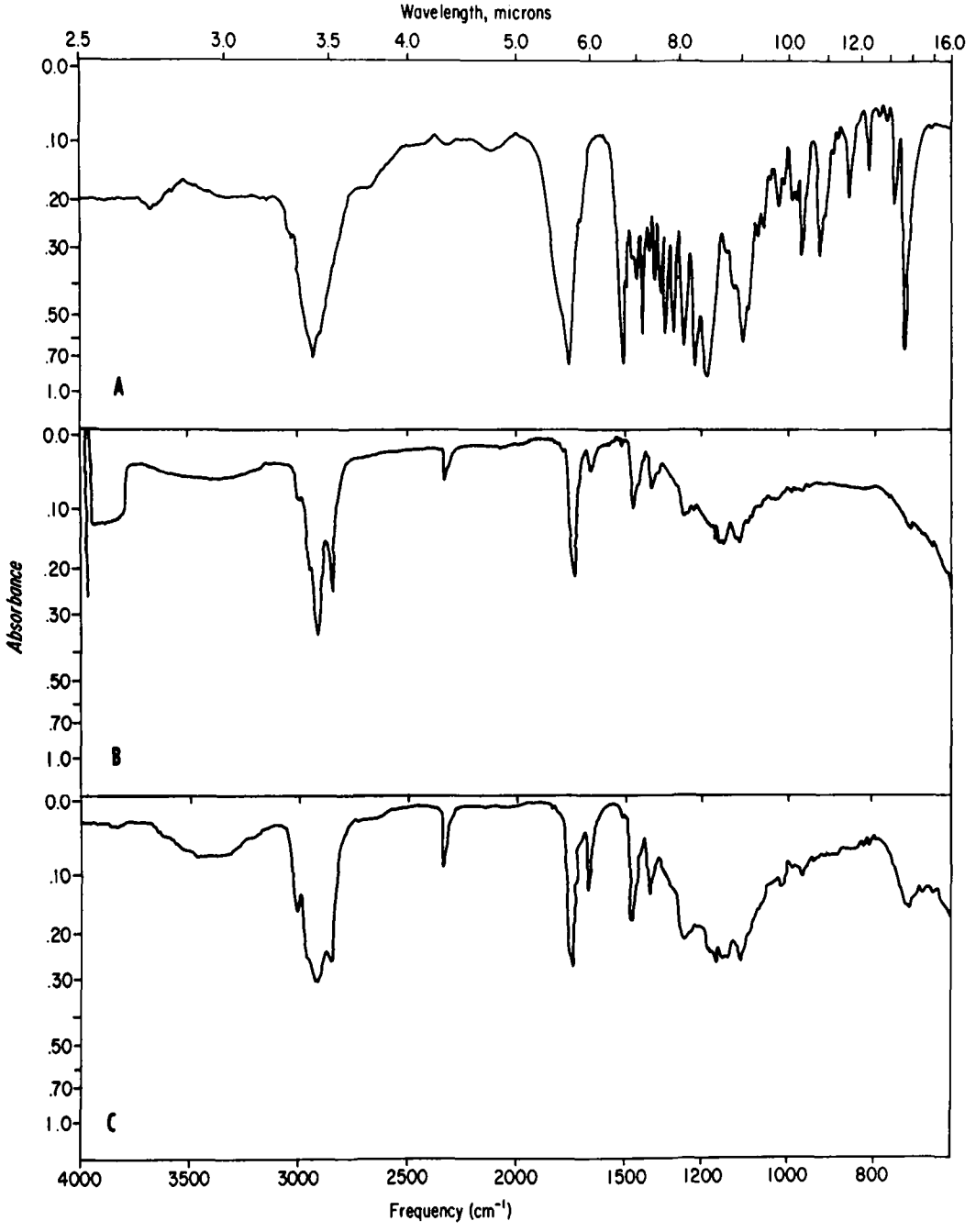

FIG. 3. Infrared spectra of (A) standard glyceryl ether diester (glyceryl-1,2-dipalmitoyl-3 hexadecyl ether), (B) glyceryl ether diester isolated from preputial gland and (C) neutral plasmalogen isolated from preputial gland.

groups (5.75μ) are present in all three spectra while the characteristic absorption (6.1μ) for vinyl ether is intense only in the plasmalogen fraction.

The hydrolysis products of the GEDE and NP and the alcohols derived from the WE fraction were analyzed by GLC. The alk-1-enyl glyceryl ethers were subjected to acid hydrolysis and the resulting aldehydes were reduced to alcohols and analyzed by GLC as

TABLE III

Per Cent Composition of Fatty Acid Methyl Esters of Four Lipid Classes[a]

Chain length and no. of double bonds	Wax %	Neutral plasmalogen %	Glyceryl ether diester %	Triglyceride %
n-C_{10}	4.3		1.5	0.8
n-C_{11}	6.3		2.0	
iso-C_{12}		3.3	1.8	
n-C_{12}	7.4	8.6	8.3	8.2
n-$C_{12:1}$			0.9	3.1
n-C_{13}	2.9	2.2	3.6	
iso-C_{14}	0.6	13.5	5.9	
n-C_{14}	0.3	7.9	6.4	2.7
n-$C_{14:1}$		3.0	3.8	2.4
n-C_{15}	3.5	2.8	8.0	
iso-C_{16}	1.3	12.6	5.1	
n-C_{16}	5.5	11.0	17.5	14.9
n-$C_{16:1}$	16.1	7.7	8.4	8.6
iso-C_{18}	7.9		3.6	
n-C_{18}	4.0		1.0	6.6
n-$C_{18:1}$	10.8	7.0	13.9	28.4
n-$C_{18:2}$	2.4		0.7	14.8
n-$C_{18:3}$	2.1			
Fatty acid Me *esters not* identified	24.6	20.4	7.6	9.5

[a]Fatty acid identification based on log plots and co-chromatography with known standards.

alcohol acetates. The alkyl glyceryl ethers were analyzed as TMS derivatives. Table II gives the percentage composition of each fraction. The saturated 14:0 and 16:0 hydrocarbon moieties predominated; only a small percentage of the hydrocarbon chains were longer than 16:0. The compositions of the alkyl and alk-1-enyl glyceryl ethers were essentially identical and clearly resembled the alcohols of the wax fraction in chain length and degree of saturation.

The fatty acids obtained after saponification of the NP, GEDE, WE and TG fractions were methylated and analyzed by GLC. Table III gives the percentage composition of the various fractions. The fatty acid composition of the TG fraction is not similar to the fatty acid composition of the other lipid classes. In addition, the fatty acid composition of the other fractions bear little similarity to each other.

This work shows that the lipids of the mouse preputial glands contain GEDE, NP and WE. The glyceryl ethers and their hydrolysis products were identified and characterized by adsorption chromatography, GLC and IR. The fatty acid, alkyl and alk-1-enyl glyceryl ether compositions of GEDE, NP, TG and WE were also determined. The composition of the aliphatic moieties of the alk-1-enyl and alkyl glyceryl ethers was shown to resemble the fatty alcohol components of the WE. These findings lend further support to the recent reports of Friedberg and Greene (4) and Ellingboe and Karnovsky (5) that long chain fatty alcohols can be direct precursors of the aliphatic chains of glyceryl ethers.

The similarities of the alk-1-enyl and alkyl glyceryl ether compositions suggest that they share a common pathway of biosynthesis or can be interconverted.

The appearance of significant quantities of branched chain and odd chain fatty acyl moieties in the NP and GEDE fractions, but not in the TG fraction, further suggests that the biosynthesis of NP and GEDE is neither related to nor shares a common biosynthetic pathway with TG.

ACKNOWLEDGMENT

This work was supported by NIH Grant No. TIGM 0648.

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[Received December 6, 1968]

Reaction of Lipoxidase With Polyenoic Acids in Marine Oils[1]

JOYCE L. BEARE-ROGERS, Research Laboratories, Food and Drug Directorate, Ottawa, Ontario, and **R. G. ACKMAN**, Halifax Laboratory, Fisheries Research Board of Canada, Halifax, Nova Scotia

ABSTRACT

Eight samples of raw oils from fresh water and marine fish, and two from marine mammals, were examined for polyenoic fatty acids susceptible to the action of lipoxidase. The results agreed well in most instances with gas chromatographic data and indicated that only one peroxide group was formed in polyenoic fatty acids with more than one ostensibly suitable 1,4-pentadiene system. Four commercially available preparations of polyenoic fatty acids showed varying degrees of susceptibility to lipoxidase, indicating the probable presence of artifacts formed in purification steps. The marine polyenoic acids were suitable substrates for lipoxidase.

INTRODUCTION

It is well established that lipoxidase catalyses the production of optically active hydroperoxides from polyenoic acids containing a *cis,cis*-1,4-pentadiene system (1,2). An early method to detect lipoxidase activity was based on the oxidation of linoleic acid and the subsequent measurement of the hydroperoxide-induced conjugation at 234 mμ (3). This procedure was modified by McGee (4) for the quantitative estimation of total *cis*-methylene-interrupted, polyenoic acids.

Hamberg and Samuelsson (5) reported the formation of both 9- and 13-hydroperoxides from linoleic acid. Dolev et al. (6) demonstrated the existence of only the 13-hydroperoxide and thereby implied a higher degree of enzymic specificity. Later work of Hamberg and Samuelsson (7) confirmed the formation of this hydroperoxide and showed that the oxygen function was introduced at the ω6 position in the fatty acids which acted as substrates, and that these were required to contain a *cis,cis*-1,4-pentadiene system with the methylene group in the ω8 position. The polyenoic C_{18}, C_{20} and C_{22} acids of marine oils reportedly possess such substrate characteristics exclusively, the C_{16} acids partially, and the odd-numbered acids hardly at all (8,10). Since no attempt appears to have been made to verify this through the action of lipoxidase, a number of raw marine oils of diverse origins and some preparations of appropriate polyenoic acids were examined.

MATERIALS AND METHODS

Substrates included: Methyl linoleate (Applied Science Laboratories); methyl arachidonate, eicosapentaenoate and docosahexaenoate (The Hormel Institute). Certain oils have been described elsewhere as follows: commercial marine flat fish oils, largely flounder, *Pseudopleuronectes americanus* (11); commercial fin whale oil, *Balaenoptera physalus* (12); herring oils, *Clupea harengus* (13); sheepshead, *Aplodinotus grunniens* and tullibee, *Coregonus artedii* (fresh water) oils (14). Cod liver oil, *Gadus morrhua* and seal oil, *Halichoerus grypus*, were obtained from the Halifax Laboratory, Fisheries Research Board of Canada, where they were analyzed by gas liquid chromatography as described elsewhere (13).

Lipoxidase obtained from Worthington Biochemicals Corp. was used at a concentration sufficient to provide a maximum ultraviolet absorption due to conjugated hydroperoxide (234 mμ) in less than 5 min, indicating completion of the enzymic reaction. Cottonseed oil, containing linoleate in triglycerides protected by the tocopherols in the oil, served as a standard substrate in checking enzymic preparations and day by day reproducibility.

Enzymic oxidation of the substrates was accomplished by the procedure of MacGee (4), adopted as follows. The methyl esters of polyenoic acids were rapidly weighed in a flask purged with nitrogen and then diluted with hexane to a concentration of approximately 1 mg/ml. Transfer to a nitrogen-purged 100 ml flask and further dilution gave a concentration of fatty acids in hexane of approximately 100 μg/ml. Aliquots (5 ml) of this solution were removed, the solvent evaporated under nitrogen, and the fat saponified. The potassium salt of the polyenoic acid (approximately 15 μg) was incubated with 30 μg lipoxidase in 0.2 M potassium borate buffer at pH 9.0 for 30 min. The absorption of conjugated hydroperoxides at 234 mμ was read against a sample containing inactivated lipoxidase. For the lipoxidase determinations on marine oils (including fresh water fish oils), 200 to 300 mg of sample was weighed into a nitrogen-purged flask and diluted to 100 ml with hexane. An aliquot (1 ml) was taken

[1]Presented at the AOCS Meeting, New York, October, 1968.

TABLE I

Percentages of *Cis*-Polyunsaturated Fatty Acids (PUFAs) as Determined by Lipoxidase and the Relative Proportions of *Trans*-Double Bonds in Commercially Available Standards

Fatty acid	*Cis*-PUFAs wt %	$A_{10.3\,\mu}/A_{5.75\,\mu}$
18:2ω6 (99%)[a]	91.9 ± 2.3[b]	(0.019)
20.4ω6 (90%)	77.6 ± 1.8	0.048
20.5ω3 (90%)	82.1 ± 0.4	0.022
22.6ω3 (99%)	68.2 ± 1.3	0.057

[a]Purity of methyl ester of fatty acid as stated on label.

[b]Mean ± SD of four determinations.

for saponification, usually overnight, as described by Zmachinski et al. (15). The enzymic incubation and determination of resulting hydroperoxide conjugation were performed as previously described for the reference lipids. Each sample was analyzed in duplicate on at least two different days with cottonseed oil of known linoleate content as a routine standard.

To detect the possible occurrence of *trans*-double bonds, the absorbance of a 1% solution of methyl esters or marine oils was determined at 10.3 μ and compared with the absorbance of the ester linkage at 5.75 μ, as described by Lees and Korn (16). Ultraviolet absorption of unreacted substrates was also checked.

RESULTS AND DISCUSSION

Lipoxidase showed various degrees of reactivity toward commercially available preparations of methyl esters of polyenoic acids (Table I). If the enzyme could react with more than one pair of methylene-interrupted double bonds per molecule of these fatty acids, there would be an apparent increase in the proportion of *cis*-polyunsaturated fatty acids. Alternatively, if only one site per molecule was involved, the proportion would not exceed the molar concentration of the particular acid. The latter was found to be the case, but the decrease from theory was greater than predicted. The purity of these standards had been determined by the supplier through gas chromatography, and apparently provided no verification that the double bonds were all *cis*-methylene interrupted. In the present study, no evidence for conjugated double bonds was obtained by absorption in the ultraviolet. In the infrared, the methyl linoleate showed some absorption, but no peak, at 10.3 μ, but the other standards exhibited small definite peaks for *trans*-double bonds. Of the two C_{20} acids, the one with the lower proportion of *cis*-isomers suitable for lipoxidase activity appeared to contain more *trans*-double bonds in unknown positions. The occurrence of *trans*-double bonds in a commercial preparation of linolenic acid was investigated by Lees and Korn (16), who isolated a mono-*trans* di-*cis*-isomer, presumably derived from the isolation processes of the all *cis*-form found in linseed oil. Mangold and Sand (17) attributed the formation of small amounts of *trans* isomers in linoleate to changes during distillation.

Raw marine oils provide sources of *cis*-polyenoic acids which have not been subjected to the complex fractionation procedures involved in the preparation of standards. Such oils may be from fish or mammals and have polyunsaturated fatty acids in proportions related to iodine value, but usually in the range of 10-40% (18). A comparison of the results of analyses for *cis*-polyunsaturated fatty acids determined by lipoxidase and by gas liquid chromatography is shown in Table II. The gas chromatographic values exclude those fatty acids which lack a double bond ω6, and consequently could not react with lipoxidase but include all linolenic types with a ω3 double bond.

For the specific sample of cod liver oil examined here, the lipoxidase value was lower than the chromatographic value. The two procedures were in fair agreement for the sample of finwhale oil and the two samples of flat fish oil. Because of the relatively large standard deviations obtained from herring samples No. 1 and 3, the confidence interval included the chromatographic values. For herring oil sample No. 6, the value by gas chromatography was markedly above the one obtained enzymatically.

The two procedures gave similar results for seal oil and tullibee oil, but the sheepshead oil was another case in which a higher value was obtained by gas chromatography. Since the lipoxidase method was standardized with linoleate in a given weight of cottonseed oil, an equivalent weight of longer chain polyenoic acids in marine oil would produce somewhat fewer conjugated hydroperoxides, but this effect would vary from oil to oil, depending on the proportions among 18:2ω6, 18:4ω3, 20:5ω3 and 22:6ω3. The general similarity in the results of the two procedures demonstrates that the relatively large number of double bonds does not increase the number of reactive sites for lipoxidase, and that most of the polyunsaturated fatty acids in these marine oils are suitable substrates for lipoxidase. Gas chromatographic errors in individual oil analyses reflect contemporary and past standards in diverse

TABLE II

Cis-Methylene-Interrupted, Polyunsaturated, Fatty Acids (PUFAs) in Marine Oils

Source of oil	*Cis*-PUFAs	
	Lipoxidase wt % of total fat	GLC wt % of methyl esters of fatty acids
Cod liver	20.7 ± 0.6 (4)[b]	24.4
Finwhale	12.2 ± 0.6 (6)	13.2 (9)[c]
Flat fish A	20.2 ± 0.8 (4)	21.6 (8)
Flat fish B	22.5 ± 0.8 (6)	23.4 (8)
Herring No. 1	11.5 ± 1.3 (4)	9.7 (10)
Herring No. 3	14.6 ± 2.1 (8)	17.2 (10)
Herring No. 6	14.8 ± 0.8 (6)	18.7 (10)
Seal	19.7 ± 0.7 (4)	20.9
Sheepshead	19.9 ± 0.6 (4)	22.4 (11)
Tullibee	22.3 ± 1.7 (6)	22.0 (11)

[a]*Cis*-PUFAs excluding those which lack a double bond at ω6.

[b]Mean ± SD (No. of determinations in brackets).

[c]Reference for complete gas liquid chromatographic analysis.

studies carried out for other purposes with a variety of apparatus and staff. The pentaenoic and hexaenoic acids from marine oils appear to react with lipoxidase in the same manner as linoleic acid, although $\Sigma\omega3/\Sigma\omega6$ ratios can be as high as 7-10 for marine oils or as low as 2-3 in fresh water fish oils (19). These findings are consistent with the early observation of Holman and Elmer (20) that linoleic, linolenic and arachidonic acids oxidized to equal degrees with lipoxidase, although their rates of autoxidation increased with unsaturation. MacGee (4) concluded that each molecule of arachidonic acid formed but one conjugated diene during the enzymic reaction.

Khalid et al. (21) recently reported that marine oils with their high degree of unsaturation were not suitable as sources of essential fatty acids. This conclusion was based on gas chromatographic data showing only traces of linoleic acid for most species tested. Evidence does exist, however, for essential fatty acid activity in the fatty acids of marine oils. Using a rat bioassay based on weight gain during water restriction, Thomasson found herring, whale and menhaden oils to contain 7.9%, 6.4% and 4.4% essential fatty acids, respectively (22), and a sample of docosahexaenoic acid to possess 20-59% of the activity found in linoleic acid (23). Another criterion, the ability to relieve the dermal symptoms of essential fatty acid deficiency, provided estimates of 2% essential fatty acids in tuna, herring and menhaden oils (24,25). Further evidence for a discrepancy between the function of an oil in promoting weight gain and in curing the dermal symptoms of essential fatty acid deficiency may be found in studies with cod liver oil (26). It has been assumed that the structural requirements for lipoxidase are also those of essential fatty acids, but apparently all lipoxidase-susceptible fatty acids do not have the same biopotency. This aspect of marine fatty acids should perhaps be reassessed. There appears to be no doubt that such oils serve as sources of polyenoic acids which may substitute in varying degrees for the conventional essential fatty acids.

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[Received December 16, 1968]

Stereospecific Analysis of the Major Triglyceride Species in the Monounsaturated Fraction of Cocoa Butter[1]

J. SAMPUGNA, Chemistry Department, University of Maryland, College Park, Maryland 20742 and
R. G. JENSEN, Department of Animal Industries, University of Connecticut, Storrs, Connecticut 06268

ABSTRACT

A stereospecific analysis employing pancreatic lipase, *Goetrichum candidum* lipase and phospholipase A was applied to the monounsaturated triglyceride fraction of cocoa butter. Diacid triglycerides required additional analyses involving the gas liquid chromatographic determination of the proportions of diglyceride acetates produced by separately acetylating the α,α and α,β-diglycerides obtained from a *G. candidum* digestion mixture. Results indicated that the major triacid triglyceride in cocoa butter was *rac* glyceryl-1-palmitate-2-oleate-3-stearate. Approximately 5% of cocoa butter was comprised of triglycerides which were solely or predominantly of one enantiomer, containing oleic acid in the 3 position.

INTRODUCTION

The development of a stereospecific analysis of triglycerides by Brockerhoff in 1965 (1) revitalized the search for enantiomeric triglycerides in natural fats. The procedure enables the separate determination of the fatty acids occupying the 1 and 3 positions and when differences are noted, these indicate the presence of enantiomeric triglycerides. However, the presence of equal amounts of the same fatty acids in both positions does not necessarily indicate the absence of specific stereoisomers. These limitations to the Brockerhoff stereospecific analysis have been discussed by Schlenk (2) and it is clear that the procedure can be utilized most effectively if the fat is first separated into triglyceride species.

Once the triglycerides are separated into species, most of the fractions are readily amenable to an analysis of the specific isomer distributions. Despite initial conclusions to the contrary (3), even the most difficult analysis of the triacid triglyceride fractions can theoretically be achieved by methods outlined by Lands and Slakey (4) and Jensen et al. (5). The latter procedure requires the presence of a fatty acid containing *cis*-9-unsaturation and is therefore the less general method; however, the scheme has recently been experimentally tested with synthetic triglycerides (6) and the results indicated that the method would be very useful in elucidating triacid triglyceride mixtures which contain oleic acid.

The procedure (6) involves a pancreatic lipase hydrolysis of 10-20 mg of substrate to determine the fatty acids in the 2 position and the incubation of another 50 mg of triglyceride with *G. candidum* lipase, which specifically splits off *cis*-9-unsaturated fatty acids (7) leaving diglycerides for further treatment. The α,α- and α,β-diglycerides are collected separately, converted to phenyl phosphatides and treated with phospholipase A. Phospholipase A specifically hydrolyzes the 1 position of 1,3 diacyl-2-*sn*-phosphatides and the 2 position of 1,2-diacyl-3-*sn*-phosphatides (8). The enzyme will not hydrolyze phospholipids which contain the phosphate group in the 1 position (8).

The analysis of the monoglycerides produced with pancreatic lipase and the analysis of the 3-monoacyl-2-*sn*-phosphatide allows the determination of the ratios of the original 2 position oleic acid triglyceride isomers, while the analysis of the 1-mono-acyl-3-*sn*-phosphatide aids in the determination of the original triglycerides which contained oleic acid in the 3 position. The 1 position oleic acid triglycerides are calculated by difference.

The monounsaturated triglyceride fraction of cocoa butter is known to contain large amounts of triacid triglycerides (2,9) therefore we chose this material to test the application of our procedure (6) to the analysis of stereospecific isomers in natural triglyceride mixtures.

Since the separation of triglyceride species in sufficient quantities for stereospecific analyses is still a tedious task we serached for additional analytical techniques to obviate the necessity for completely separating the monounsaturated triglyceride fraction. The analysis of diglyceride acetates synthesized from the diglycerides resulting from *G. candidum* lipolysis seemed especially useful, thus this technique was utilized to supplement our original procedure (6). These techniques have been applied to the monounsaturated triglyceride fraction of cocoa butter and the results are reported in this paper.

The numbering used in this manuscript is according to the rules for stereospecific numbering as outlined in the IUPAC-IUB Commission on Biochemical Nomenclature, The Nomencla-

[1]Scientific contribution No. 348 Agricultural Experiment Station, University of Connecticut, Storrs.

ture of Lipids (10). In this system, the glycerol molecule is viewed in a Fischer projection formula with the secondary hydroxyl (or derived group) to the left and the hydrogen on the asymmetric carbon to the right. Then, the carbinol group at the top is position 1 and the group at the bottom is position 3. When stereospecific numbering is indicated the term signifying glycerol is prefixed with *sn*; where racemic glycerides are intended by the written formula, the term signifying glycerol is prefixed with *rac*. Also in this paper, an abbreviated representation of triglyceride nomenclature is used and when stereospecific numbering is intended, the first fatty acid depicted is in position 1 while the last fatty acid mentioned is in position 3. For example, *sn*-PSO is used as an abbreviation for *sn*-glyceryl-1-palmitate-2-stearate-3-oleate. In the abbreviated formula, *rac*-PSO, palmitic and oleic acids are considered to occupy the 1 and 3 positions equally. Where the Greek letters α and β are employed, no knowledge of optical isomerism is intended; thus in these instances racemates, enantiomers or partial racemates could be involved.

MATERIALS AND METHODS

Lipases

The *G. candidum* lipase was prepared as described by Alford and Smith (11). In this study the phospholipase A was *Crotalus atrox* venom (Ross Allen Reptile Institute, Silver Springs, Fla.) and the purified pancreatic lipase (Worthington Biochemical Corporation, Freehold, N.J.) contained 100 units/mg.

Isolation of Monounsaturated Triglycerides From Cocoa Butter

Cocoa butter (U.S.P.) (Fisher Scientific Co., Medford, Mass.) was chromatographed on a column of alumina to remove polar constituents (12). The monounsaturated triglyceride fraction was isolated by preparative silver nitrate-thin layer chromatography (TLC), using chromatographic plates coated with silicAR TLC-7G (Mallinckrodt Chemical, New York) containing 15.5% by weight of silver nitrate (based on the dry adsorbent). Five milliliters of a 0.01% ethanolic solution of Rhodamine G was included in the slurry to facilitate subsequent visualization. The plates were coated to a thickness of 250 μ and allowed to dry at room temperature for ca. 15 min before activation at 100 C for 1 hr. Activated plates were stored in a desiccator over $CaSO_4$ until used.

The TLC plates (ca. 100) were charged with ca. 25 mg of cocoa butter triglycerides dissolved in chloroform and developed in a chloroform-methanol (98:2) solvent system until the solvent front had travelled half way up the plate. The plates were allowed to dry, then developed further in a solvent system of absolute chloroform until the solvent front had reached the top of the plate. The monounsaturated band was visualized under UV light, scraped into a flask and stored at -12 C. When sufficient material had been collected for an analysis, the lipid was extracted from the adsorbent with chloroform-methanol (90:10). The extract was concentrated and the lipid purified by preparative TLC on layers of Silica Gel G 500 μ thick. Development was in a solvent system of petroleum ether (bp 35-45 C)-ethyl ether (85:15) and the triglyceride band was located by visualization under UV light without the aid of indicators, as the band was quite concentrated. The triglyceride band was extracted from the TLC adsorbent with petroleum ether (bp 35-45 C) and the solvent removed under a stream of nitrogen.

Enzymatic Procedures

The purified triglyceride mixture was emulsified in 1% gum arabic and incubated for 15 min at 37 C with *G. Candidum* lipase. The lipid was extracted from the digestion mixture with ethyl ether and the diglycerides in the mixture isolated by preparative TLC on 500 μ layers of Silica Gel G containing 5% by weight (based on the dry adsorbent) of boric acid (13). Development was in a chloroform-acetone (96:4) solvent system and the diglyceride bands were located with the aid of iodine vapors. The separately collected α,α-diglycerides and α,β-diglycerides were converted to phosphatidyl phenols and these were subsequently incubated with phospholipase A. The products resulting from this digestion were isolated by preparative TLC, converted to methyl esters and analyzed by gas liquid chromatography (GLC). Complete details on all of the above procedures and of the pancreatic lipolysis method are contained in a previous publication (6).

Preparation of Diglyceride Acetates

The diglycerides to be analyzed (25-50 μmoles) were placed in a flask and 0.5 ml of pyridine and 0.5 ml of acetic anhydride were added. The flask was stoppered and the reagents were allowed to react overnight. The reaction mixture was transferred to a separatory funnel with 50 ml of chloroform-methanol (2:1). Twenty milliliters of water were added, the contents shaken and the phases allowed to separate. The organic phase was reduced in volume, streaked on a preparative TLC plate coated to a thickness of 500 μ with Silica Gel G

and developed in a petroleum ether (bp 35-45 C)-ethyl ether-acetic acid (90:30:1) solvent system. The diglyceride acetate band was located by brief exposure to iodine vapors and eluted from the adsorbent with petroleum ether (bp 35-45 C). The solvent was reduced under a stream of nitrogen to a suitable volume prior to analysis of the diglyceride acetates by GLC.

Analytical Procedures

Four aliquots of the monounsaturated fraction from cocoa butter triglycerides were digested with *G. candidum* lipase; one sample was used for the diglyceride acetate analysis and three samples were converted to phenyl phosphatides and analyzed with phospholipase A. Controls containing no substrate were carried through the entire analytical procedure. To facilitate determination of relative proportions between samples, a known amount of pentadecanoic acid was added prior to the TLC separation of the products from phospholipase A hydrolysis. Methyl heptadecanoate was added prior to esterification to aid in the calculation of the relative proportions of fractions within a sample.

The original cocoa butter triglycerides, the monounsaturated triglyceride fraction and each of the fractions isolated after phospholipase A hydrolysis were analyzed as methyl esters by GLC. In addition the monounsaturated fraction was analyzed by GLC of the intact triglycerides.

Methyl esters were separated on 10 ft by 0.25 in. stainless steel columns containing 18% Hi Eff DEGS coated on Anakrom ABS 70/80 mesh (Analabs, North Haven, Conn.). Columns were operated at 195 C in a model 5000 Selecta System (Barber Colman Co., Rockford, Ill.) employing dual flame detectors. A series 200 instrument (Varian-Aerograph, Walnut Creek, Calif.) equipped with dual flame detectors was employed to analyze the triglycerides and diglyceride acetates. These compounds were separated on 2 ft by 1/8 in. stainless steel columns packed with 2% OV-1 coated on Anakrom Q, 100/120 mesh (Analabs, North Haven, Conn.). Triglycerides were separated at a temperature of 325 C while the diglyceride acetates were analyzed at 280 C. Disc integrators (Disc Instruments, Inc., Santa Ana, Calif.) attached to the recorders were used to quantitate the areas under the peaks.

Detector response to the triglycerides was determined by standards prepared from the synthetic triglycerides, *rac*-OPP, *rac*-OSP and *rac*-OSS. Similarly, standards of diglyceride acetates were prepared by weighing quantities of the synthetic diglycerides, *rac*-glyceryl-1,3-dipalmitate, *rac*-glyceryl-1-oleate-3-palmitate and *rac*-glyceryl-1-oleate-3-stearate and converting these mixtures to diglyceride acetates. All of the glycerides were synthesized in our laboratory (14). The relative recoveries of the diglyceride acetates were quantitative within experimental error (a relative error of 5% for major peaks and 10% for minor peaks) however, it was necessary to correct for apparent losses of *rac*-OSS in the analysis of triglycerides.

The logic behind the calculation of the specific glyceride isomers involves a knowledge of the specificity of *G. candidum* lipase and phospholipase A. Detailed calculations are contained in a previous publication (6) and modifications employed in this paper as a result of the use of the diglyceride acetates are discussed below.

RESULTS AND DISCUSSION

Based on the GLC determination of the methyl esters of the fractions isolated by silver nitrate TLC, the monounsaturated fraction constituted 74% of the total triglycerides. The trisaturated and polyunsaturated triglyceride fractions were respectively 2.2% and 23.8% of the total. In Table I are presented the fatty acid compositions of the total triglycerides in cocoa butter, of the monounsaturated fraction isolated by silver nitrate TLC and the monoglycerides derived from pancreatic lipolysis of the monounsaturated triglyceride fraction. In general the values were in close agreement with those reported previously (15). Also presented in this Table are the results from the phospholipase A analysis of the phosphatidyl phenols synthesized from the diglycerides isolated from *G. candidum* hydrolysis. Two important points which will be more fully considered subsequently were the absence of palmitic acid in the 2,3-diacyl-1-*sn*-phosphatides remaining after phospholipase A analysis and the percentage of 2,3-diacyl-1-*sn*-phosphatide remaining after digestion (13.6%).

The analysis of the monounsaturated fraction by GLC of the intact triglycerides gave evidence for at least three major triglyceride species with carbon numbers (these refer only to the carbons in the acyloxy groups) of 50, 52 and 54. The 52 carbon peak, which was the triacid triglyceride fraction of interest, constituted 48.6% of the triglycerides while the 50 and 54 carbon species contained respectively 18.2% and 33.2% of the material.

Based on these results it was considered virtually impossible to isolate the triacid triglyceride fraction in quantities sufficient for a precise stereospecific analysis according to the procedure outlined in our previous paper (6);

TABLE I

Fatty Acid Composition and Percentage of the Total Triglycerides of Cocoa Butter, the Monounsaturated Triglycerides Isolated From Cocoa Butter and Some of the Fractions Resulting From the Enzymatic Analysis of the Monounsaturated Fraction[a]

Fraction	Fatty acids, mole %						Per cent of total TG represented
	14:0	16:0	18:0	18:1	18:2	20:0	
Intact TG	0.20	27.7	31.8	35.9	3.6	0.8	100.0
S_2U TG	Trace	27.0	38.0	34.4	----	0.6	74.0
S_2U MG[b]	Trace	3.6	3.1	93.3	----	Trace	74.0
3-Mono-acyl-2-*sn*-phosphatide[c]	----	41.0	59.0	----	----	Trace	69.0[d]
1-Mono-acyl-3-*sn*-phosphatide[c]	----	31.6	68.4	----	----	----	4.3[e]
2,3-Diacyl-1-*sn*-phosphatide[c]	----	0.0	100.0	----	----	----	0.7[e]

[a]TG, triglyceride, MG, monoglyceride, S_2U, monounsaturated triglyceride fraction isolated as described in text.

[b]Derived from pancreatic lipase analysis.

[c]Derived from the phospholipase A analysis of the phenyl phosphatides synthesized from the diglycerides obtained from *G. candidum* lipolysis.

[d]Based on the percentage of oleic acid in the monoglyceride.

[e]Based on the percentage of oleic acid in the alpha positions and the per cent hydrolysis of the alpha phosphatides (86.4%).

therefore, a combination of methods was employed. First, a portion of the monounsaturated fraction was incubated with *G. candidum* lipase, the 'α,α- and α,β-diglycerides were separately isolated, converted to diglyceride acetates and analyzed as such by GLC. A second portion of the fraction was analyzed with the aid of *G. candidum* and phospholipase A as described (6). The results from the latter analysis are shown in Table I and have already been referred to. The results from the diglyceride acetate analysis are displayed in Table II along with other data utilized to calculate the isomer proportions present in the monounsaturated fraction from cocoa butter.

The elucidation of the isomer proportions in the original 2 position oleate triglycerides was relatively simple since the amounts of glyceryl-2-oleate-1,3-dipalmitate (POP) and glyceryl-2-oleate-1,3-distearate (SOS) were immediately available from the diglyceride acetate analysis (Table II). Based on the phospholipase A analysis of the 1,3-diacyl-2-*sn*-phosphatides (Table I), 41.0% of the 2-oleate fraction consisted of POP and *sn*-SOP, as both of these glycerides would have contributed palmitate to the 3-mono-acyl-2-*sn*-phosphatide fraction if 16:0 was present. As the amount of POP was known (17.5%), subtraction allowed the calculation of *sn*-SOP (23.5%). In an analogous fashion the proportions of SOS and *sn*-POS were determined to be, 35.0 and 24.0 mole %.

The procedure for determining the glycerides containing oleic acid in the alpha positions was not as simple. Theoretically, this fraction could have been resolved by performing, in addition to the determinations described above, further analyses of the 2,3-diacyl-1-*sn*-phosphatide (non-hydrolyzed alpha phosphatide) as diglyceride acetates, to determine the proportions of *sn*-OPP, *sn*-OPS, *sn*-OSP and *sn*-OSS. If this fraction (2,3-diacyl-1-*sn*-phosphatide) could have been converted to diglyceride acetates, by the cumulative result of phospholipase C and acetylation and subsequently analyzed with pancreatic lipase, resolution should have been possible. However, the amount of material available in this fraction was insufficient to warrant testing this hypothesis. Furthermore, the data presented in Tables I and II were sufficient to calculate each of the isomers present in the mixture because of the absence of palmitic acid in the 2,3-diacyl-1-*sn*-phosphatide (Table I). The absence of palmitic acid in this fraction indicated that there was no palmitic acid in the original triglycerides which contained oleic acid in *sn* position 1, as these glycerides would have been converted to 1-sn-phosphatidyl phenols which would not have been hydrolyzed by phospholipase A and would have remained as residual 2,3-diacyl-1-*sn*-phosphatide. Therefore, there was no *sn*-OPP, *sn*-OPS and *sn*-OSP or conversely the entire fraction of 1 position oleate triglycerides consisted of *sn*-OSS. With this additional information, the determination of the other isomers was considerably simplified.

From the diglyceride acetate data the amount of *sn*-SSO and *sn*-OSS was observed to be 30.6% (Table II). Since the percentage of 2,3-diacyl-1-*sn*-phosphatide (13.6%) represent only *sn*-OSS, the percentage of this enantiomer was calculated by subtraction.

TABLE II

Estimated Isomer Proportions in the Monounsaturated Triglyceride Fraction of Cocoa Butter

Glyceride	Mole % Based on diglyceride acetates[a]	Based on phosphatides[a]	Calculated	Total glycerides
POP	17.5	41.0[b]	17.5	12.1
sn-SOP	47.5		23.5	16.3
sn-POS		59.0[b]	24.0	16.5
SOS	35.0		35.0	24.2
sn-OPP	21.3	0.0[c]	0.0	0.0
sn-PPO		27.3[d]	21.3	1.1
sn-PSO			6.0	0.3
sn-OSP	48.1	0.0[c]	0.0	0.0
sn-OPS		0.0[c]	0.0	0.0
sn-SPO		59.1[d]	42.1	2.1
sn-SSO	30.6		17.0	0.8
sn-OSS		13.6[e]	13.6	0.7

[a]The first four glycerides are calculated as a percentage of the β-unsaturated α,α-disaturated fraction while the rest of the glycerides are percentages of the α-unsaturated-α,β-disaturated triglycerides.

[b]Based on the percentage of palmitate and stearate in the 3-mono-acyl-2-*sn*-phosphatides.

[c]Based on the absence of palmitate in the 2,3-diacyl-1-*sn*-phosphatides.

[d]Based on the amounts of palmitate and stearate found in the 1-mono-acyl-3-*sn*-phosphatides and the per cent hydrolysis of alpha phosphatides.

[e]Based on the amount of stearate found in the 2,3-diacyl-1-*sn*-phosphatides and the per cent hydrolysis of the alpha phosphatides.

From the diglyceride acetate information and the absence of palmitic acid in the 2,3-diacyl-1-*sn*-phosphatide, a value of 21.3% was obtained for *sn*-PPO. The palmitate in the total alpha phosphatides (27.3%), represented the percentage of *sn*-PPO and *sn*-PSO combined as both of these compounds would have formed 3-*sn*-phosphatidyl phenols, which when hydrolyzed with phospholipase A would yield lysophosphatides containing palmitic acid. Therefore, the amount of *sn*-PSO was 27.3% - 21.3% or 6.0%. The amount of *sn*-SPO (42.1%) was obtained from the diglyceride acetate value, for *sn*-PSO, *sn*-OSP, *sn*-OPS and *sn*-SPO (48.1%), the knowledge that two of these isomers were absent and the previously calculated *sn*-POS percentage (48.1 - 6 = 42.1).

The values in the last column of Table II have been normalized to reflect the amounts of each of the isomers which occurred in the original cocoa butter. The virtual absence of triglycerides containing oleic acid in position 1 is striking. It should be noted, however, that the highest value for the α-unsaturated, α,β-disaturated isomers was only 2.1%, consequently it was difficult to assess the significance of these values. As a total, these compounds represented 6.7% of the monounsaturated fraction and only 5% of the total triglycerides.

The values obtained for the triacid triglyceride pair in the β-unsaturated α,α-disaturated fraction indicated that this triglyceride was racemic and confirmed the results of Schlenk (2) who arrived at the same conclusions using x-ray diffraction data. In other respects the percentages of the major isomers, including the diacid triglyceride species, were in general agreement with those reported by Jurriens and Kroesen (15).

ACKNOWLEDGMENTS

Supported in part by Public Health Service Research Grant AM-02605-10 from the Institute of Arthritis and Metabolic Diseases. Mrs. Linda Mitchell, Jane Rosesco and R.M.P. Caley collected the monounsaturated fraction of cocoa butter. *G. candidum* lipase was a gift from J. A. Alford, USDA, Beltsville, Md.

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[Received November 25, 1968]

New Sources of 9-D-Hydroxy-*cis*-12-octadecenoic Acid[1]

R. G. POWELL, R. KLEIMAN and **C. R. SMITH, JR.,**
Northern Regional Research Laboratory,[2] Peoria, Illinois 61604

ABSTRACT

9-D-Hydroxy-*cis*-12-octadecenoic acid has been isolated from 3 seed oils of the family Apocynaceae: *Holarrhena antidysenterica* (73%), *Nerium oleander* (11%) and *Nerium indicum* (8%). The known occurrence of this acid was previously limited to the genus *Strophanthus* (9-15%). A mixture of unusual tetra-acid glycerides was isolated from *N. oleander* oil by thin layer chromatography. Pancreatic lipase hydrolysis of the glyceride mixture showed that 9-D-acetoxy-*cis*-12-octadecenoic acid is esterified exclusively at an α-glycerol position and that normal fatty acids occupy the remaining 2 glycerol positions. A portion of the hydroxy acid in *N. indicum* oil was also acetylated, however, no acetate was found in either *H. antidysenterica* or *Strophanthus hispidus* oils.

INTRODUCTION

Gunstone (1) first reported the occurrence of 9-hydroxy-*cis*-12-octadecenoic acid in *Strophanthus sarmentosus* seed oil and later showed that it probably is of general occurrence (9-15%) in seed oils of the genus *Strophanthus*, family Apocynaceae (2). The structure has been confirmed by synthesis (3) and the naturally occurring "Strophanthus acid" has been assigned the D configuration (4). The *Strophanthus* acid may also be obtained by partial diimide reduction of naturally occurring 9-D-hydroxy *trans*-10,*cis*-12-octadecadienoic acid (5,6).

Strophanthus oils are convenient sources of minor amounts of 9-D-hydroxy-*cis*-12-octadecenoic acid, however, the low hydroxy acid content and unavailability of the oils suggested a search for additional sources among the closely related genera *Holarrhena* and *Nerium*.

In this paper we report the characterization of 9-D-hydroxy-*cis*-12-octadecenoic acid from 3 new sources. One, *Holarrhena antidysenterica* (Roth) Wall ex. DC. seed oil, contains this acid as the major component (73%). We also report the characterization of an unusual tetra-acid glyceride mixture isolated from *Nerium oleander* L. seed oil.

PROCEDURES AND DATA

Infrared (IR) spectra were determined on a Perkin-Elmer 137 instrument as liquid films or as 1% solutions in carbon tetrachloride or carbon disulfide. Nuclear magnetic resonance (NMR) spectra were recorded on Varian A-60 or HA-100 spectrometers with deuteriochloroform solutions containing 1% tetramethylsilane as an internal standard (unless otherwise indicated). Optical rotatory dispersion (ORD) measurements were made on a Cary Model 60 recording spectropolarimeter at 26° in absolute methanol and in 0.5 dm cells. Melting points were determined with a Fisher-Johns melting point apparatus and are uncorrected.

Thin layer chromatography (TLC) was performed on plates coated with 0.25 mm or 1.0 mm layers of Silica Gel G or 20% silver nitrate-impregnated Silica Gel G with 20% or 30% ether in hexane as the solvent. Boric acid-impregnated plates were used for analysis of lipolysis products. Spots on analytical plates were visualized by charring with sulfuric acid-chromic acid. After preparative plates were sprayed with dichlorofluorescein, bands were visualized under ultraviolet light.

Gas liquid chromatography (GLC) of methyl esters was conducted as described by Miwa et al. (7). Triglycerides were analyzed on an F&M Model 5750 instrument equipped with a 24 X 1/8 in. metal column packed with 3% OV-1. The instrument was linearly temperature-programmed at 4 deg/min from 200-400 C.

Oxidative cleavages were according to the method of von Rudloff (8). Iodine values were determined by the AOCS method (9). Pancreatic lipase hydrolyses, silylations and subsequent GLC of silylated lipolysis products were as described by Tallent and Kleiman (10).

Isolation and Purification of Hydroxy Acids

Seeds were ground and extracted overnight in a Soxhlet apparatus with petroleum ether (30-60 C); solvent was removed from the oils on a rotary evaporator. Both *Nerium oleander* and *Nerium indicum* oils exhibited bands in the IR at 1235 cm^{-1} and 1020 cm^{-1} (acetate) which were not present in spectra of *H. antidysenterica* or *Strophanthus hispidus* oils.

Methyl esters were prepared by refluxing the oils for 2 hr with 5% hydrochloric acid in

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[1]Presented at the AOCS Meeting, San Francisco, April, 1969.

[2]No. Utiliz. Res. Dev. Div., ARS, USDA.

anhydrous methanol. Esters were recovered by ether extraction and were analyzed by GLC. Oil yields, iodine values and analyses of the methyl esters by GLC are recorded in Table I.

Concentrates of the hydroxy esters were obtained by preparative TLC. The concentrates were saponified by refluxing for 1 hr with 1N potassium hydroxide in ethanol, and unsaponifiables were removed by extracting dilute aqueous solutions of the soaps with ether. After acidification, the free hydroxy acids were recovered by ether extraction. The acids were esterified with diazomethane, and the resulting esters were purified by preparative TLC. Overall yields were near 80% in each instance (based on GLC of the total esters).

Characterization of Hydroxy Acids

Hydroxy acid methyl esters from the 4 oils (*H. antidysenterica, N. oleander, N. indicum*, and *S. hispidus*) were indistinguishable by IR and NMR. The spectra were consistent with a C_{18} methyl ester having 1 hydroxyl and 1 *cis* double bond. GLC of the esters gave single peaks, > 98%, for each and retention characteristics of all 4 esters were identical. The esters showed single spots which were not differentiated by TLC on either ordinary or silver-nitrate impregnated plates.

Samples of hydroxy ester from each of the 4 oils were cleaved oxidatively and methyl esters of the products were prepared by refluxing with acidic methanol. The IR spectrum of each oxidation product had a strong band at 1773 cm^{-1} characteristic of a γ-lactone, and GLC showed methyl hexanoate and a fragment corresponding to a C_{12} γ-lactone-methyl ester (5). Thus, the hydroxy acids from all 4 sources are C_{18} acids having hydroxyl at C_9 and a *cis* double bond at C_{12}. ORD curves were also obtained for each of the 4 methyl ester samples (Table II).

Hydrogenation of the hydroxy ester from *H. antidysenterica*, with palladium-charcoal in hexane, gave methyl 9-hydroxyoctadecanoate. After preparative TLC and recrystallization from hexane, the saturated ester melted at 51-52 C and had a plain negative ORD curve (Table II). Known methyl 9-D-hydroxyoctadecanoate gives a similar melting point, 51.5-52.8 C, and a nearly superimposable plain negative ORD curve (11).

A 156 mg portion of methyl 9-hydroxy-*cis*-12-octadecenoate, from *Holarrhena* oil, was acetylated by allowing to stand overnight in a mixture of pyridine-acetic anhydride (1:1). Column chromatography on 2 g of neutral alumina (hexane) yielded 176 mg of product. The product gave IR and NMR spectra which

TABLE I

Analytical Data on Selected Apocynaceae Seed Oils

Source	Oil, % in seed	Iodine value		Composition of methyl esters, % (area percentage by GLC)							
		Wijs	Calc.	16:0	16:1	18:0	18:1	18:2	18:3	Hydroxy 18:1	Others
Holarrhena antidysenterica	33.7	134.4	83.2	5.0	---	2.7	7.7	7.8	1.3	73.4	2.1
Nerium oleander	29.4	114.1	115.8	9.3	0.1	4.7	24.8	47.1	1.3	10.6	2.1
Nerium indicum	26.1	117.2	121.9	9.2	0.1	5.6	22.0	51.7	0.2	7.7	3.5
Strophanthus hispidus	33.8	---	---	13.6	---	8.1	38.0	29.3	0.1	10.3	0.6

TABLE II

Optical Rotatory Dispersion of Hydroxy Esters

Ester and source	Specific rotation in degrees $[\alpha]_{589}$	$[\alpha]_{500}$	$[\alpha]_{400}$	Concentration, g/100 ml
Methyl 9-D-hydroxy-*cis*-12-octadecenoate				
Holarrhena antidysenterica	-0.83	-1.18	-2.00	2.88
Nerium oleander	-1.76	-2.30	-3.20	1.13
Nerium indicum	-1.92	-2.34	-3.40	0.95
Strophanthus hispidus	-1.87	-2.48	-4.12	2.67
Strophanthus kombe[a]	-1.17	-1.76	-2.54	1.02
Methyl 9-D-hydroxyoctadecanoate				
Hydrogenated *Holarrhena* ester	-0.12	-0.16	-0.30	2.29
Hydrogenated methyl dimorphecolate[a]	-0.17	-0.23	-0.37	1.49

[a]Specific rotations of these materials were obtained by Applewhite et al. (11) and are listed here for comparison.

were consistent with those expected for methyl 9-acetoxy-*cis*-12-octadecenoate. GLC and TLC showed the product to be homogeneous.

Glyceride Characterization

An unknown component was observed upon TLC analysis of *N. oleander* oil, which had an R_f slightly lower than normal triglycerides. GLC of the oil indicated that acetotriglycerides and triterpene acetates were absent because no significant components with carbon numbers less than C_{52} were observed. A significant peak was eluted as C_{56}, however, even though only 2% C_{20} esters were found in the ester analysis. The presence of glycerides containing an acetylated hydroxy acid constituent was indicated by a conspicuous 1235 cm^{-1} (acetate) band in the IR; only weak hydroxyl absorption was present.

IR of the unusual component, isolated by preparative TLC, showed a strong 1235 cm^{-1} (acetate) band and no hydroxyl. A sharp singlet was present in the NMR spectrum at τ 8.1 (CCl_4 solution) characteristic of an acetoxy group. A multiplet was evident at τ 5.3, due to a single proton, and a similar multiplet was noted in the NMR spectrum of methyl 9-acetoxy-12-octadecenoate. This signal was assigned to the proton on a carbon bearing an acetoxy group (C_9). A multiplet, equivalent to 4 protons, was present at τ 5.9, indicative of protons on the outer carbons of a glycerol moiety. Except for the signals at τ 8.1 and τ 5.3, the spectrum was otherwise consistent with that of a glyceride. This unusual component, when analyzed by GLC, gave peaks having carbon numbers of C_{52}, C_{54} and C_{56}.

After the unusual glyceride mixture was subjected to pancreatic lipase hydrolysis, a product was isolated that migrated between normal free acids and diglycerides on TLC. This product had an IR band at 1705 cm^{-1}, associated with free carboxylic acid, but also had bands at 1724 cm^{-1} and 1235 cm^{-1}, characteristic of an acetoxy derivative. Treatment with diazomethane gave the methyl ester which was identical with known methyl 9-acetoxy-*cis*-12-octadecenoate, as demonstrated by IR, NMR, GLC and TLC.

A portion of lipolysate of the unusual glyceride mixture was silylated, and GLC showed no monoglycerides with acetoxy functional groups. Similar treatment of a lipolysate of the original oil showed that 5% of the monoglycerides contained acyl groups with hydroxy or acetoxy functions, or both. A portion of the whole oil lipolysate was treated with diazomethane and subsequent GLC demonstrated that 12% acetoxy and 2% hydroxy methyl esters were present in the mixture. Thus, assuming no acyl migration during lipolysis, all the acetoxyacyl and a portion of the hydroxyacyl groups are esterified on the outer glycerol carbons.

DISCUSSION

The hydroxy acids of *H. antidysenterica, N. oleander, N. indicum* and *S. hispidus* are clearly identical, and experimental data are consistent with only one structure: 9-D-hydroxy-*cis*-12-octadecenoic acid. The presence of this acid in *S. hispidus* oil was demonstrated previously (12). Assignment of the D configuration was possible as these 4 esters and known methyl 9-D-hydroxy-*cis*-12-octadecenoate (11) all gave plain negative ORD curves that were nearly superimposable. Confirmation of this assignment was obtained by hydrogenating the *Holarrhena* ester; the product gave an ORD curve essentially the same as that for known methyl 9-D-hydroxyoctadecanoate (4,11).

Gunstone (1) found that methyl 9-hydroxy-12-octadecenoate gives abnormally high iodine

values, apparently because of involvement of the reagent with the hydroxyl group. Similarly, the iodine value determined for *Holarrhena* oil (Table I) is considerably higher than that calculated from the fatty acid composition. More satisfactory results may be obtained after acetylation (1) and the *Nerium* oils do not show this discrepancy.

Since pancreatic lipase (EC 3.1.1.3) displays a strong specificity for the 1 and 3 (or α and α') positions of triglycerides (13), lipolysis with this enzyme has been widely used to determine the intraglyceride distribution of various acyl groups. In *Strophanthus* oils 9-hydroxy-12-octadecenoic acid shows strong preference for the 2 (or β) glycerol position, and there is no evidence for triglycerides with more than 3 acyl groups (14). Since lipolysis of the mixture of tetra-acid glycerides from *N. oleander* gave no monoglycerides with acetoxy functional groups, evidently 9-acetoxy-12-octadecenoic acid occurs exclusively at 1 (or both) of the α glycerol positions. However, 9-hydroxy-12-octadecenoic acid is also present, as shown by lipolysis of the whole oil, and the major portion of this acid occurs at the β glycerol position. The tetra-acid glycerides of *N. oleander*, containing an acetylated monohydroxy acid, are apparently unique although similar tetra-acid (15) and penta-acid (16) glycerides having acetylated di- or trihydroxy acids are also known.

The *Holarrhena* alkaloids are considered possible starting materials for industrial preparation of steroid hormones, including the adrenocortical hormone aldosterone (17). Oils from other species of *Holarrhena* may prove equally rich in 9-hydroxy-12-octadecenoic acid, and moderate quantities of the oils could become available as by-products of the pharmaceutical industry. At present, *H. antidysenterica* seed oil is, by far, the richest known source of 9-D-hydroxy-*cis*-12-octadecenoic acid.

ACKNOWLEDGMENTS

Preliminary screening results and GLC analyses by F. R. Earle, J. W. Hagemann and R. W. Miller; NMR spectra by L. W. Tjarks; *Holarrhena* and *Nerium* seeds were furnished by Q. Jones, USDA, Beltsville, Md., and the *Strophanthus* seeds by F. D. Gunstone, University of St. Andrews, Scotland.

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[Received April 3, 1969]

Acceleration and Inhibition of Lipid Oxidation by Heme Compounds

JEAN KENDRICK and **BETTY M. WATTS**, Department of Food and Nutrition, Florida State University, Tallahassee, Florida 32306

ABSTRACT

The acceleration and inhibition of unsaturated fatty acid oxidation by heme compounds was followed in model systems with an oxygen analyzer. The linoleate to heme molar ratios for maximum catalysis were 100 for hemin and catalase, 250 for metmyoglobin, 400 for cytochrome c and 500 for methemoglobin. With heme concentrations of 2 to 4 times the optimum catalytic amount, no oxidation occurred. Rapid heme destruction was observed with catalyzing ratios of lipid to heme, but with inhibitory ratios a stable red compound formed, believed to be a lipid hydroperoxide derivative of the heme. The ratios of lipid to metmyoglobin for maximum acceleration varied with the pH. Linolenate was much less sensitive to heme catalysis than linoleate. Colorless products of heme degradation had a marked antioxidant effect. A possible mechanism for the antioxidant effect of hemes is advanced, based on the formation of stable heme peroxide complexes or stable heme radicals, or both, during the early stages of oxidation. Prooxidant activity is believed to occur only when the peroxide to heme ratio is so high that the oxidation of the hemes goes beyond the initial stages.

INTRODUCTION

The catalytic effect of iron porphyrins on the oxidative decomposition of polyunsaturated fatty acids was first described by Robinson in 1924 (1). Some of the voluminous work on this important biocatalyst has been reviewed by Tappel (2). The mechanism proposed by Tappel for the prooxidant activity of hemes is based on their known ability to decompose lipid peroxides. New reaction chains are assumed to be initiated by free radicals resulting from the peroxide scission, although direct attack by the heme catalyst on the fatty acid is not ruled out.

That heme compounds can act as antioxidants rather than prooxidants under some conditions was recognized much more recently. Maier and Tappel in 1959 (3), using a Hb catalyst, found that when the concentration of linoleate dropped below a specified level, prolonged induction periods occurred. They attributed this to too little peroxide in the reaction mixture to generate sufficient free radicals for chain initiation. Apparently they did not consider the possibility that Hb itself was acting as an antioxidant.

Banks et al. (4), using increasing amounts of cytochrome c (cyt c) with fixed amounts of fatty acid, found increasing acceleration up to a maximum and then progressive inhibition with still higher concentrations of cyt c. They postulated that the peroxide decomposition brought about by hemes did not result in chain initiating radicals but rather in stable end products and that cyt c became a prooxidant only after being altered in some way by reaction with the lipid peroxides.

Perhaps the most extensive and convincing evidence to date of the antioxidant activity of heme compounds is that of Lewis and Wills (5). These workers demonstrated that Hb, cyt c, hematin and tissue homogenates at high concentrations all had an inhibitory effect on linoleate oxidation, whereas at lower concentrations they catalyzed the oxidation. The concentration of Hb necessary to show antioxidant activity increased with higher concentration of fatty acid. Thus the ratio of Hb to fatty acid determined whether acceleration or inhibition would take place. In the inhibitory range, Hb stopped fatty acid oxidations, catalyzed by cobalt, which were already well under way. No mechanism was suggested.

The obvious implications of these concentration related differences in the effect of heme compounds on lipid oxidation in living tissues and in meats and fish has led us to explore their activity further. By measuring the rate of lipid oxidation with various heme compounds under the same experimental conditions, concentrations for peak acceleration and inhibition could be compared and the relative magnitude of the accelerating effects evaluated. Observations have also been made on changes in heme compounds when brought into contact with linoleate in catalyzing versus inhibiting concentrations and the effect of the decomposed hemes on lipid oxidation.

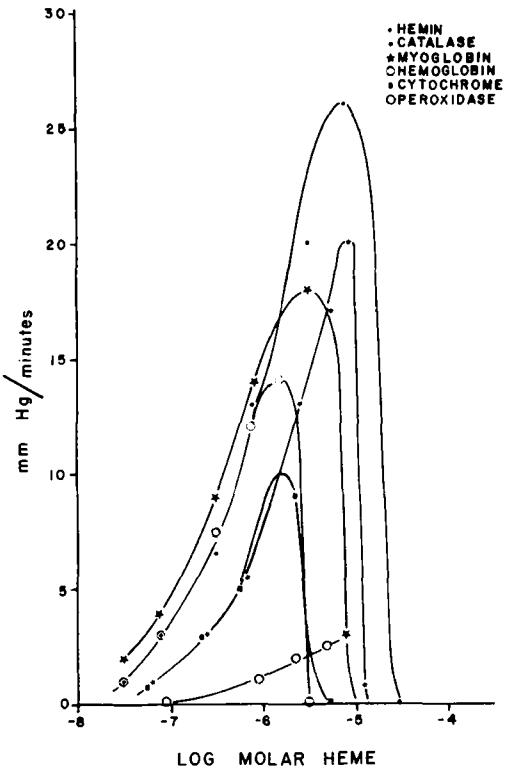

FIG. 1. The effect of varying concentrations of heme compounds on linoleate oxidation, pH 7.4; linoleate concentration, 8.0 x 10^{-4} M.

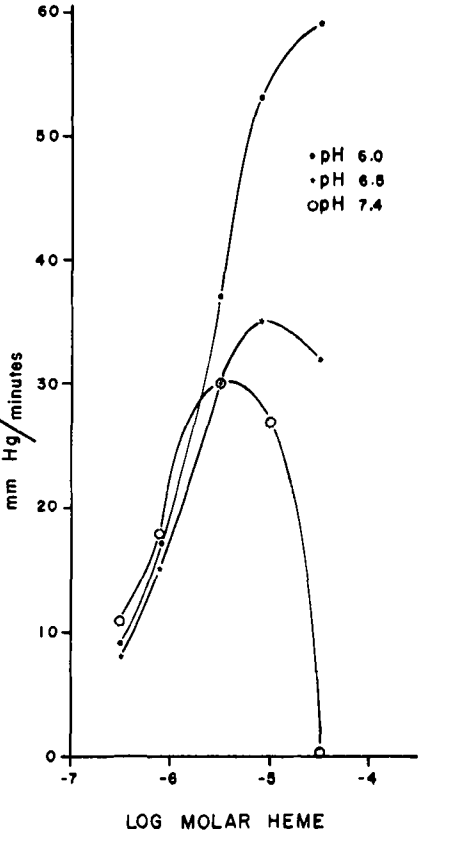

FIG. 2. The effect of pH on the catalytic activity of metMb. Linoleic acid, 8.0 x 10^{-4} M.

MATERIALS AND METHODS

Heme Compounds

Nutritional Biochemicals Corporation supplied hemin (recrystallized), horse metMb (once crystallized and lyophilized), horse radish peroxidase and beef liver catalase (lyophilized). MetHb, bovine (twice crystallized, A Grade) and cyt c, horse heart (salt free, A grade) were obtained from Calbiochem. The methemoglobin (metHb), metmyoglobin (metMb), hemin and cyt c were put into solution at pH 8.5 with M/10 phosphate buffer, the pH adjusted to 7.4 and the solution made to volume with M/10 phosphate buffer, pH 7.4. The peroxidase and catalase were dissolved directly with pH 7.4 buffer.

All of the compounds used were tested by the Hornsey method (6) for acid hematin and all molarities corrected to the equivalent heme basis. The heme contents of the metHb, metMb, cyt c, catalase and peroxidase were 100%, 100%, 68%, 31% and 29% respectively of the theoretical values.

Fatty Acid Emulsions

Linoleic acid (LA) and linolenic acid (LNA) were obtained from the Hormel Institute. Clear aqueous emulsions of the fatty acids were prepared as described by Surrey (7) and adjusted to the concentration and pH desired with M/10 phosphate buffer.

Oxygen consumption in the model systems was measured with a Beckman Model 777 oxygen analyzer. The fatty acid emulsions (43 ml) were transferred to 50 ml Erlenmeyer flasks and stirred magnetically at room temperature. The desired amount of buffered heme compound, contained in 5 ml, was added with a fast delivery pipette. The sensor of the oxygen analyzer was immediately inserted. From the linear slope of the recording, the rate of oxygen consumption by the system was determined and expressed as change in mm Hg PO_2 min. Neither the fatty acids nor the heme compounds oxidized at a measurable rate when tested separately.

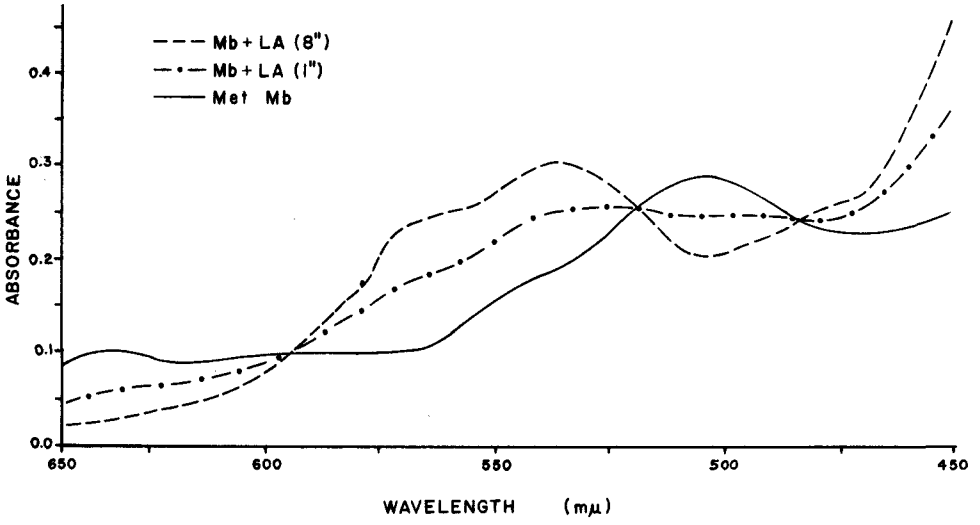

FIG. 3. Changes in the absorption spectrum of metMb when brought into contact with linoleic acid. MetMb, 3.2 x 10^{-5} M.; linoleic acid, 8 x 10^{-4} M; pH 7.4.

RESULTS

Effect of Varying Heme Concentrations

The comparative effect of various heme compounds on the oxidation of linoleate is shown in Figure 1. The peroxidase had only a slight effect at the concentrations tried and was not explored further. Increasing concentrations of all of the other heme compounds progressively increased lipid oxidation up to a maximum value. Further increases in concentration resulted in a sharp drop in lipid oxidation to zero. Uncombined hemin had the greatest range of activity and the highest rate before an inhibitory concentration was reached. All of the protein linked hemes fell within this range.

The data for each heme compound in Figure 1 were obtained on different days, using different linoleate emulsions. When hemin, metMb and metHb were tested at lower concentrations on the same fatty acid emulsion, the initial slopes of all curves were identical, within experimental error. Thus, these three compounds differ in their catalytic action only in that inhibition begins at progressively lower heme concentrations with metHb, metMb and hemin respectively. Catalase and cyt c also resemble each other in initial slopes, but differ in peak concentrations.

The approximate molar ratios of linoleate to heme at the point of maximum catalytic effect (Fig. 1) were 100 for hemin and catalase, 250 for MetMb, 400 for cyt c and 500 for MetHb. At heme concentrations ranging from 2 to 4 times those giving maximum acceleration, oxygen consumption fell off to zero.

Changes With pH

The effect of varying the pH was tried on hemin and metMb. The concentration of hemin giving maximum acceleration was the same at pH 7.4, 6.7 and 6.1 and the general form of the curve was not changed. However, the maximum rate of oxidation increased as the pH decreased. With metMb, not only the rate of oxidation but also the heme concentrations at maximum acceleration increased with decreasing pH (Fig. 2). The crossing over of the curves is a real phenomenon, not attributable to experimental error, i.e., at low metMb concentrations, the highest rate invariably occurs at the highest pH, although the differences are not great.

Linolenic vs. Linoleic Acid

When linolenic acid was substituted for linoleic, again the heme compounds (hemin, metMb and metHb) showed increased catalytic action up to maximal acceleration, followed by inhibition at higher concentrations. The same concentrations of the above heme compounds were maximally effective with linolenic acid as with linoleic, but the concentrations of linolenic acid had to be increased at least five times over that of linoleic to get comparable oxidation rates. The oxidation with 8 x 10^{-4} M linolenic acid was not measurable. A typical set of data with metMb as catalyst is shown in Table I. Obviously the lipid to heme ratio at maximum acceleration will be five times higher for linolenic acid than for linoleic. Tappel (2) pointed out the much lower ratio of heme catalyzed oxidation to autoxidation in linolenic versus linoleic acid.

TABLE I

Effect of MetMb on Linoleic vs. Linolenic Acid

MetMb Concentration (molar)	O_2 Uptake in mm Hg/min Linoleate 8×10^{-4} M	Linolenate 4×10^{-3} M
8.0×10^{-8}	4.0	1.4
3.2×10^{-7}	9.0	3.5
8.0×10^{-7}	14.0	10.5
3.2×10^{-6}	18.0	13.5
8.0×10^{-6}	3.0	5.5
3.2×10^{-5}	0.0	0.0

TABLE II

Inhibition of Linoleate Oxidation by Heme Degradation Products[a]

Hemes degraded	Inhibition of linoleate oxidation, %
MetHb, 6.7×10^{-6} M	52
MetHb, 6.7×10^{-7} M	39
MetMb, 5.5×10^{-6} M	72
MetMb, 5.5×10^{-7} M	60
Hemin, 5.5×10^{-6} M	73
Hemin, 5.5×10^{-7} M	52

[a]Linoleic acid, 7.2×10^{-4} M, catalyzed by 2.9×10^{-6} M hemin at p^H 7.4.

Observations on Pigment Changes

When catalyzing concentrations of heme compounds were brought into contact with linoleic acid, destruction of the hemes invariably occurred within a few minutes, with complete loss of all peaks in the visible and Soret regions of the spectrum. The concomitant oxidative destruction of the heme catalyst during oxidation of unsaturated fats has been described repeatedly in the literature.

On the other hand, with inhibiting concentrations of heme compounds, new colored reaction products formed upon contact with linoleic acid. These were studied spectrophotometrically with metMb and metHb. With metMb, the brown color changed to red within a few minutes after the addition of the linoleic acid emulsion. Figure 3 shows the changes in the absorption spectrum during the first 8 min. There was only a slight further development of the new peaks at 535 and 563 mμ after an additional 30 min. The new pigment was quite stable; the red color was still apparent when the reaction mixture was held in the refrigerator overnight. Suitable inhibitory ratios of metHb and linoleic acid also produced a red pigment with a spectrum very similar to that of the metMb reaction product. When the red pigments were diluted with linoleic acid emulsion to bring the lipid and heme concentrations into an accelerating ratio, the pigments were quickly destroyed and the lipid oxidized.

The formation of such red pigments in the presence of ferric hemes and lipid hydroperoxides was described by Tappel (8). He interprets the pigments as denatured globin hemichromes. In view of the large concentrations of various nitrogen bases required for hemichrome formation, an alternative interpretation of the pigment as a complex between the ferric hemes and lipid hydroperoxides seems more likely. The formation of such red complexes between H_2O_2 and MetHb (9) and cyt c peroxidase (10) has been described.

Antioxidant Effect of Heme Oxidation Products

The effect of heme breakdown products on lipid oxidation was tested as follows: hydrogen peroxide was added slowly to solutions of hemin, metHb and metMb until the Soret band could no longer be observed. Approximately 2 ml of 30% H_2O_2 were required to decompose 50 ml of 10^{-5} M heme. The solution resulting from hemin oxidation was clear and colorless; those from metHb and metMb were slightly cloudy after treatment, denoting some denaturation.

Neither the oxidized solutions nor H_2O_2 accelerated oxidation of fresh linoleic acid preparations. When added to rapidly oxidizing linoleic acid, catalyzed by hemin, the decomposed heme products inhibited the oxidation. At concentrations sufficiently high to bring the heme lipid ratio above the normal accelerating range for fresh hemes, the decomposed products brought about complete inhibition. Dilutions of the solutions containing the oxidized products gave partial protection (Table II). Since the antioxidant effect was as great with the oxidation products of hemin as with those of metHb or metMb, it can be ascribed to fragments of the porphyrin ring rather than to the globins. The antioxidant effect of iron free porphyrins has been pointed out by Matsushita and Iwami (11).

DISCUSSION

The data presented here and in the more recent literature suggest that heme compounds act initially as antioxidants, combining with lipid peroxides, as they are produced, to form relatively stable compounds, thus retarding the initiation of new reaction chains. It is only in the presence of large amounts of peroxide relative to the heme that the pigment is altered and free radical reactions are initiated.

The protein moiety confers further stabilizing effects upon the conjugated hemes. Hemin itself had the least antioxidant activity, i.e., the ratio of lipid to heme necessary to overwhelm the heme and convert it to a prooxidant, was generally higher with protein bound hemes (Fig. 1).

King and Winfield (12) and King et al. (13) have pointed out the complex nature of the oxidation of metMb by H_2O_2. Loss of electrons occurs both from iron and from easily oxidizable aromatic amino acids of the protein in the vicinity of the heme. The initial free radical with quadrivalent iron (Mb IV) is relatively stable and capable of combining with other free radicals in the system. Thus it could stop reaction chains in an oxidizing fatty acid system but presumably lacks sufficient energy to initiate new chains by abstracting H from fresh fats. Probably only at a later stage of oxidation, when the porphyrin ring itself is decomposing, are radical intermediates formed of sufficient energy to initiate new reaction chains in oxidizing lipids.

The abrupt shift from antioxidant to maximally active prooxidant with relatively slight increases in the lipid to heme ratios, noted with all of the heme compounds tested (Fig. 1), is rather surprising. The transitory nature of the catalyzing intermediates of the oxidizing hemes and the fact that the end products as well as the initial products of their oxidation are antioxidant in character, probably contribute to this behavior. When the heme concentration reaches a critical level relative to lipid peroxide formation, energetic radicals, which may result from destruction of a fraction of the heme, are removed by reaction with stable heme radicals such as Mb IV and the degraded porphyrin ring eventually supplements the antioxidant action. At lipid to heme ratios below this critical value, the heme is able to contain the free radicals formed, with the result that lipid oxidation is retarded indefinitely. At high ratios the reaction quickly gets out of hand, with wide spread heme destruction and rapid acceleration of lipid oxidation.

The critical ratios of lipid to heme may be expected to vary with the system. The state of dispersion of the unsaturated fat and the heme in heterogeneous systems would certainly influence such ratios, since only the fat in contact with the heme could influence the ratio. A significant pH effect, similar to that noted for metMb (Fig. 2) might also be expected to occur with other conjugated hemes. The relative insensitivity of linolenic as compared to linoleic acid is worthy of further exploration. A more thorough and detailed study of the intermediates formed with each of the hemes and the peroxides of the common unsaturated fatty acids is obviously needed.

Considering the complex structural relationships in animal tissues, speculations as to the probable effect of cellular hemes on lipid oxidation are not highly profitable. Treatments such as the cooking or freezing of flesh foods might be expected to influence such ratios drastically.

ACKNOWLEDGMENT

This study was supported by Agricultural Research Service, U.S. Department of Agriculture, Grant No. 12-14-100-7655(73), administered by the Eastern Utilization Research and Development Division, Philadelphia, Pennsylvania 19118.

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[Received December 12, 1968]

Lecithin-Protein Interaction in the GTP-Dependent Acyl-CoA Synthase[1]

LAURO GALZIGNA, LODOVICO SARTORELLI, CARLO R. ROSSI,
The Institute of Biological Chemistry, University of Padova, Padova, Italy and
DAVID M. GIBSON, Department of Biochemistry, Indiana University School of Medicine, Indianapolis, Indiana 46202

ABSTRACT

The enzymic activity of the GTP-dependent acyl-CoA synthase isolated from rat liver mitochondria is affected by removal and addition of lecithin (3). Purified preparations of this enzyme contain bound lecithin. In this presentation the nature of the lecithin-protein interaction is examined. From binding measurements at different temperatures it is possible to postulate which type of secondary valence bonds is holding the protein and lecithin together.

INTRODUCTION

Two GTP-dependent acyl-CoA synthase preparations have been described, both catalyzing the reaction:

$$R\text{-}COOH + CoASH + GTP \rightleftharpoons R\text{-}COSCoA + GDP + P_i$$

where RCOOH stands for fatty acids; CoASH, reduced coenzyme A; GDP and GTP, guanosine di- and triphosphates, respectively; P_i, inorganic orthophosphate. The first was isolated from acetone powders of beef liver mitochondria (1) and was active with fatty acids up to 12 carbons. The second was prepared from sonically-disrupted rat liver mitochondria and accepted both short and long chain fatty acid substrates (2). Subsequent work demonstrated that lecithin may play an important role in determining the catalytic activity of the second enzyme (3). Purified preparations of this enzyme contained lecithin. Successive extraction of the lyophilized enzyme with 90% acetone diminished enzyme activity. The activity was almost completely restored by adding back the extracted lecithin or purified egg lecithin (3).

The sensitivity to the treatment with organic solvent could explain the differences in substrate specificity between the two enzymes. The beef liver synthase, active on short chain fatty acids only, was purified from an acetone powder of beef liver mitochondria. No organic solvent was used during the purification of rat liver synthase. In other words, endogenous, bound lecithin seemed to impose the chain length specificity.

In the present communication the recombination of enzyme protein with lecithin is further quantitated in order to evaluate the nature of this interaction.

MATERIALS AND METHODS

Enzyme activity was routinely assayed with oleate as substrate by measuring CoA sulfhydryl group disappearance and inorganic phosphate formation (2). The incubation mixture was the same as previously described (2). Incubations were carried out under nitrogen for 10 min at 38 C.

The GTP-dependent acyl-CoA synthase was isolated from rat liver mitochondria following procedures previously described (2). The purified enzyme was lyophilized and 16 mg of protein were extracted three times with 10 ml aliquots of 90% acetone at room temperature. Lecithin was purified from egg phospholipids by thin layer chromatography (4). The recombination of the acetone-extracted protein with a relative excess of lecithin was carried out by incubating 0.8 mg of protein with 0.5 mg of lecithin in 0.2 ml of 0.1 M Tris-buffer (pH 7.4) for 30 min. The molar ratio of lecithin to protein, 40:1, was obtained using 20,000 as the molecular weight of the enzyme (2) (Fig. 1).

RESULTS

Only 21% of the initial specific activity remained after extraction of the native enzyme with acetone (Table I). The activity could be restored to a maximum of 80% of the initial full activity by preincubating the acetone-extracted protein for 30 min at 0 C with egg lecithin. By increasing the temperature of incubation, a smaller per cent of the initial full activity was restored (Fig. 1).

On the assumption that the resultant enzymic activity is a function of the percentage of protein saturated with lecithin, thermodynamic calculations on the binding can be made. The Arrhenius law is satistied (Fig. 1). By resolving the Van't Hoff relation, an

[1]Presented at AOCS Meeting, Chicago, October 1967.

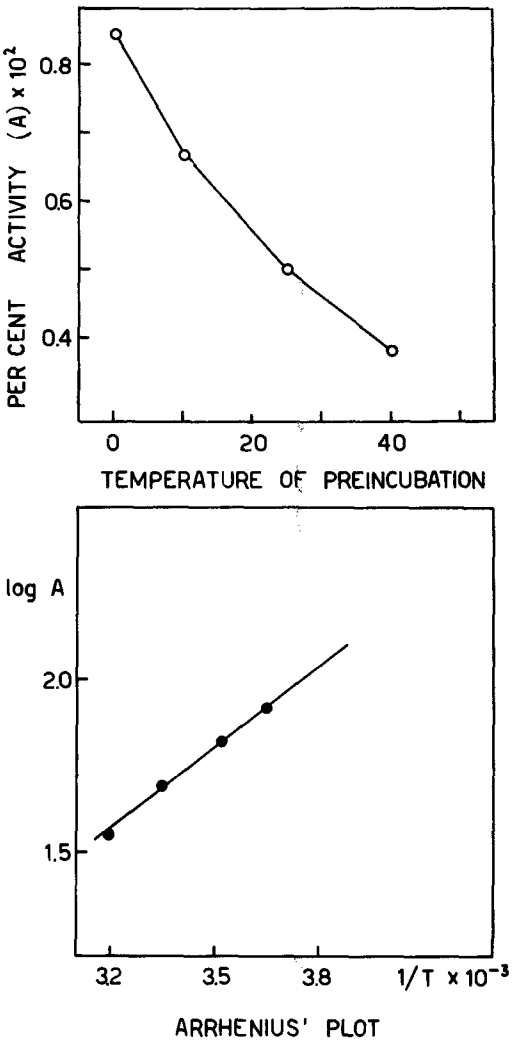

FIG. 1. Top; Per cent of original enzyme activity (A) vs. temperature of preincubation with lecithin. Lecithin was added to the acetone-extracted protein at a molar ratio of 40:1. The samples were preincubated at the temperatures indicated for 30 min and then assayed as described (2); Bottom; Arrhenius plot, per cent of enzyme activity (A) vs. the reciprocal of the absolute temperature of incubation.

enthalpy change ΔH (negative value) of about -3 kcal/mole and an entropy change ΔS (negative value) of about -12 entropy units have been found. The calculation of the thermodynamic parameters is made by using the following equations:

$$\Delta H = 4.57\ T_1T_2\ \log(A_2/A_1)\ /\ (T_2-T_1) \text{ and}$$
$$\Delta S = 4.57\ (T_2\ \log A_2 - T_1\ \log K_1)\ /\ (T_2-T_1)$$

where A is the per cent of initial enzyme activity and T is the absolute temperature.

TABLE I

Effect of Lecithin on the Activity of the GTP-Dependent Acyl-CoA Synthase[a]

Enzyme	Specific activity
Untreated	5.00
Acetone extracted-protein	1.05
Acetone extracted-protein + lecithin	4.00

[a]Specific activity of the rat liver mitochondrial GTP-dependent acyl-CoA synthase extracted three times with acetone. Recombination was carried out by preincubation of extracted protein with lecithin for 30 min at 0 C (see Methods and Materials). The molar ratio of lecithin to protein was 40:1. Specific enzyme activities are expressed in terms of μmoles of -SH disappearance per hour per milligram of protein.

DISCUSSION

In view of the fact that the extraction of lecithin from the GTP-dependent acyl-CoA synthase can be achieved by using organic solvents under mild conditions, it seems probable that secondary valence bonds are involved between the enzyme protein and the phospholipid molecule. However, the binding of the phospholipid to the protein has the features of an exothermic reaction which gives rise to a lipid-protein complex. The small negative entropy indicates that the complex has a lower number of degrees of freedom, which suggests the possibility of electrostatic bonds between the polar groups of lecithin and the polar groups of protein. The solvation of both of the reacting species (and hydrogen bonding) may regulate water structure and thereby influence the entropy change. In any case the small negative value of the entropy change is more characteristic of the ionization of dipolar ions.

In the GTP-dependent acyl-CoA synthase, lecithin, linked to the protein by electrostatic bonds, may confer chain length specificity through its hydrophobic residues. In other words, long chain fatty acids (in contrast to short chain fatty acids) need the hydrophobic residues of lecithin to facilitate their binding to the enzyme, perhaps through conformational adaptivity of the active sites of the enzyme. Such a conformational adaptivity of binding sites, put forward by Karush in model systems (5), is in agreement with the features of the superstructural components of biological membranes (6).

The binding of phospholipids with mitochondrial enzymes has been discussed extensively by Green and Fleischer (7), who emphasized two types of interaction occurring in mitochondria. The first is mainly hydro-

phobic in nature and involves the interaction of structural protein with phospholipids. The second is ionic and occurs between acidic phospholipids, but not lecithin, and basic proteins such as cytochrome c. The GTP-dependent acyl-CoA synthase may represent a new class of lecithin-protein binding.

ACKNOWLEDGMENT

This investigation was supported, in part, by grants from U.S. Public Health Service (HE 04219-10, HE 06308, TW 00104-03), the American Heart Association (67-700), the American Cancer Society (P-178I), and the Consiglio Nazionale delle Ricerche d'Italia.

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[Received January 7, 1969]

Teucrium depressum Seed Oil: A New Source of Fatty Acids With Δ^5-Unsaturation

C. R. SMITH, JR., R. M. FREIDINGER,[1] J. W. HAGEMANN, G. F. SPENCER and I. A. WOLFF, Northern Regional Research Laboratory,[2] Peoria, Illinois 61604

ABSTRACT

The seed oil of *Teucrium depressum* Small yields two unusual trienoid components, all-*cis*-5,9,12-octadecatrienoic acid (6.7%) and *trans*-5,*cis*-9,*cis*-12-octadecatrienoic acid (2.0%). A third unusual component, identified as *cis*-5,*cis*-9-octadecadienoic acid, also occurs in this oil as a trace constituent.

INTRODUCTION

Teucrium is a genus of herbaceous plants in the family Labiatae. Bailey (1) describes them as "herbs, subshrubs or shrubs, some of which are greenhouse plants or grown outdoors in the extreme South, others of them hardy in the North and suitable for the wild-garden or rockwork." About 160 species occur throughout the warmer and temperate areas of the world (1).

Previous work at this Laboratory has shown that many seed oils of the plant family Labiatae (mint family) contain unusual fatty acids, particularly those with allenic groupings (2-4). Screening results indicated that the seed oil of *Teucrium depressum* contains unusual fatty acids that are not allenes (4). This paper describes the isolation and characterization of these components.

EXPERIMENTAL PROCEDURES AND RESULTS

General Methods

Esterifications and transesterifications were carried out according to the following procedures. Samples were refluxed 1 hr in a large excess of methanol containing 1% sulfuric acid (v/v). In each case, resulting mixtures were diluted to the cloud point with water, chilled in an ice bath, and then extracted repeatedly with petroleum ether. Combined extracts were dried over sodium sulfate and evaporated in vacuo.

Analytical thin layer chromatography (TLC) was performed on plates coated with 20% silver nitrate-impregnated silica, as described by Barrett et al. (5). For preparative TLC, layers 1 mm thick were used. Either benzene or benzene-diethyl ether mixtures were used as developing solvents. The preparative plates were impregnated with dichlorofluorescein as an internal indicator, and bands were visualized under ultraviolet (UV) light. Spots on the analytical plates were visualized by charring with sulfuric acid-chromic acid.

Infrared (IR) spectra were determined with a Perkin-Elmer Model 137 instrument on 1% solutions in carbon tetrachloride or carbon disulfide. Nuclear magnetic resonance (NMR) spectra were obtained with a Varian HA-100 spectrometer on deuterochloroform solutions.

Permanganate-periodate oxidations were carried out by von Rudloff's method, specifically according to the modification in which *t*-butyl alcohol is used as a cosolvent (6).

Gas liquid chromatographic (GLC) analyses of methyl esters were performed by the method of Miwa et al. (7). GLC analyses of ozonolysis products were conducted by the method of Kleiman et al. (8).

Preparations of Mixed Methyl Esters

Coarsely ground seeds of *Teucrium depressum* Small (38.0 g) were extracted for 12 hr in a Soxhlet apparatus with petroleum ether. Upon evaporation of the solvent, 9.12 g of oil was obtained.

An 8.7 g portion of the oil was converted to a mixture of methyl esters by acid-catalyzed transesterification. According to GLC analysis, these esters had the composition indicated in Table I. Examination of these esters by TLC on silver nitrate-impregnated silica revealed distinct spots attributable to saturates, monoenes, dienes and trienes. A *trans*-double bond absorption (10.35 μ), relatively weak in intensity, was observed in the IR spectrum of these esters.

Countercurrent Distribution of Methyl Esters

Countercurrent distribution (CCD) of mixed methyl esters from *Teucrium depressum* seed oil was carried out with an acetonitrile-hexane system by the general procedure of Scholfield et al. (9). An 8.42 g sample of the esters was divided among the first eight tubes of a 200 tube Craig-Post apparatus. Ten milliliters of upper phase and 40 ml of lower phase were used throughout the distribution. After the 200 fundamental transfers were completed, upper phases were decanted into a fraction collector, two transfers being placed in each tube. Six

[1]Student trainee, 1965-1968.

[2]No. Utiliz. Res. Dev. Div., ARS, USDA.

TABLE I

GLC Analysis of Mixed Methyl Esters Derived From *Teucrium depressum* Seed Oil[a]

Ester[b]	ECL[c] on R-446 column	Percentage
$C_{16}S$	16.00	8.8
$C_{18}S$	18.00	5.9
$C_{18}I$	18.33	25.6
$C_{18}II$	18.91	48.3
$C_{18}III$ (unusual)	19.18	8.7[d]
$C_{18}III$ (linolenate)	19.68	0.5

[a]Area percentage of methyl ester.

[b]S, saturated; I, one double bond; II, two double bonds; III, three double bonds.

[c]ECL, equivalent chain length (7).

[d]This figure includes all-*cis*-5,9,12-octadecatrienoate (6.7%) and *trans*-5,*cis*-9,*cis*-12-octadecatrienoate (2.0%), the relative amounts of which were determined by TLC.

hundred transfers were applied. The weight distribution achieved was as follows: Tubes 0-20, 0.310 g; 21-55, 2.10 g; 56-95, 4.40 g; 96-160, 0.950 g. Results of GLC analysis (R-446 column) of selected fractions are summarized in Table II.

Isolation of Two Isomeric C_{18}-Trienoic Esters by Preparative TLC

Esters removed from CCD tubes 100, 110, 120 and 130 were examined by TLC on silver nitrate-impregnated silica plates (analytical) with the solvent system benzene-ethyl ether (3:1). Only two spots, corresponding to the isomeric *cis* and *trans* C_{18} trienes, were revealed by charring; these were moderately well separated. No separate spot for ordinary linolenate was observed. The best preparative separations were obtained by using 1 mm thick silica plates, activated at 120 C, with the solvent system benzene-ether (3:1). In a typical example, 69.3 mg of triene concentrate, derived from CCD tubes 101-103, was applied to a plate. The faster moving of the rather broad bands yielded a product (12.1 mg) that was 84.1% *trans* by quantitative IR analysis (10) and 94.3% pure by GLC, while the slower moving band provided a *cis*-isomer (49.3 mg) 97.8% pure by GLC. The all-*cis* triene was not resolved from the *trans,cis, cis*-isomer in GLC. The small amount of linolenate in the mixture probably overlapped both bands obtained by TLC. The NMR spectra of the two esters are nearly identical; both have a quartet at τ8.3 associated with Δ^5-unsaturation.

Hydrogenation of Mixed Triene Methyl Esters

A mixture (16 mg) of the compounds derived from the triene region of the CCD was hydrogenated with a platinum oxide catalyst at 1 atm and room temperature. The hydrogenated product, isolated by filtration and evaporation, was a white solid. It contained 99.5% methyl stearate as determined by GLC.

Microozonolysis of Triene Isomers

Samples of both the *cis* and *trans* triene fractions from TLC were each subjected to

TABLE II

Composition of CCD Fractions[a]

Tube number[b]	Transfer[c]	Ester[d]				
		$C_{16}S$	$C_{18}S$	$C_{18}I$	$C_{18}II$	$C_{18}III$
18	236	0.4	92.7			
39	278	26.2		73.8		
50[e]	300	7.7	0.5	85.6	5.0	
55[f]	310	1.5		37.8	50.8	
60[g]	320			3.5	89.4	
80	360				100.0	
100	400				24.0	75.5[h]
130	460					100.0[i]

[a]Results determined by GLC and expressed as percentage; some minor components are omitted or indicated as footnotes. ECL values in footnotes refer to the R-446 column.

[b]Numbers of tubes used to collect upper phases. Two were combined in each tube.

[c]Number of transfers completed when upper phase was introduced into the tube indicated.

[d]S, saturated; I, one double bond; II, two double bonds; III, three double bonds.

[e]The monoene peak had a shoulder corresponding to ECL 18.6.

[f]This tube also contained 3.5% of ECL 16.6 and 5.4% of ECL 18.7.

[g]This tube also contained 3.0% of ECL 16.6 and 3.8% of ECL 18.6.

[h]75.2% ECL 19.2 and 0.3% ECL 19.8 by GLC.

[i]85.0% ECL 19.2 and 15.0% ECL 19.7 by GLC.

TABLE III

Fragments From Microozonolysis of C_{18} Trienes

Fragment[a]	Area %	
	Cis-isomer[b]	*Trans*-isomer[c]
6 A	6.50	9.51
4 AA + 9:1 A	6.19	7.84
5 AE	11.08	14.26
9 AE	0.86	1.45
9:1 AE	2.20	3.22
Unreacted ester (5,9,12-isomer)	63.54	60.20
Unreacted ester (9,12,15-isomer)	4.93	2.12

[a]A, aldehyde (monofunctional); AA, dialdehyde; AE, aldehydo-ester; unsaturation, if any, is indicated by numeral after colon.

[b]Ozonized for 60 sec.

[c]Ozonized for 50 sec.

reductive ozonolysis. GLC analysis of products isolated after 20, 50 and 110 sec (*trans*-isomer) or 30, 60 and 120 sec (*cis*-isomer) showed nearly identical fragments (Table III).

Partial Hydrazine Reduction of trans C_{18} Triene; Isolation and Characterization of Reduction Products

A 59.4 mg portion of pure *trans* C_{18} triene was dissolved in 15 ml of absolute ethanol; 0.05 ml of 64% hydrazine was added, and the mixture was sparged with air, kept at 54 C for 31 hr, and then at room temperature for 15 hr. The reaction was terminated by acidification to pH 1 with hydrochloric acid. The reduction products were isolated by repeated extraction with diethyl ether. Combined, dried, ether extracts were evaporated, and the residue was chromatographed with benzene as the developing solvent on activated preparative silica plates impregnated with silver nitrate. Separated bands were removed from the plate by means of a Goldrick-Hirsch aspirator (11). Samples eluted from the silica with ethyl ether and isolated by evaporation of solvent in vacuo are described in Table IV.

The *trans* monoene was located in band II by a combination of IR and GLC analyses. This band was contaminated with a plasticizer from Tygon tubing. Microozonolysis of the sample showed the following cleavage products: C_5 aldehydo-ester, 9.91%; C_{13} aldehyde, 51.26%. The plasticizer (determined to be octyl phthalate) did not interfere with the analysis although a number of extraneous, unidentified degradation products were recorded. The *cis*-monoenes appeared in band III. Microozonolysis of the band showed the following cleavage products: C_6 aldehyde, 6.72%; C_9 aldehyde, 13.15%; C_9 aldehydo-ester, 11.66%; C_{12} aldehydo-ester, 15.08%.

Isolation of an Unusual Diene

A third unusual constituent of the oil having an equivalent chain length (ECL) of about 18.6 (R-446) was found in minor amounts in the CCD fractions that represented the trailing edge of the ordinary monoene and leading edge of the ordinary diene peak (Table II). Preparative TLC with activated plates and benzene-ether (3:1) as the developing solvent yielded only a few milligrams of the desired compound. The IR spectrum of the compound showed no peak at 10.35 μ corresponding to *trans* unsaturation. Microozonolysis afforded the following cleavage products: C_5 aldehydo-ester, 3.46%; C_9 aldehyde, 12.38%; C_9 unsaturated aldehydo-ester, 1.82%; C_{13} unsaturated aldehyde, 1.30%. Other products were detected, including 34.30% of starting material.

TABLE IV

Fractions Isolated by Preparative TLC After Hydrazine Reduction of *Trans* C_{18} Triene[a]

Band	Weight (mg)	Composition[b] (by GLC)	ECL of major components (R-446 column)[c]
I	6.1	12.4% C_{18}S[d]	18.02
II	29.6	64.3% C_{18}I	18.33
III	6.4	87.0% C_{18}I	18.35, 18.45
IV	5.9	95 % C_{18}II	18.80
V	5.9	94 % C_{18}II	18.75 (35.9%), 18.99 (58.5%)
VI	4.1		
VII	11.8		

[a]In order of decreasing R_f.

[b]S, saturated; I, one double bond; II, two double bonds.

[c]ECL, equivalent chain length.

[d]This band is about 70% plasticizer accidentally introduced from Tygon tubing; percentages shown here are based on components other than plasticizer.

DISCUSSION

The unusual triene fraction derived from *Teucrium* seed oil proved to be a mixture of two components, one shown by IR to have a *trans* double bond and another with all-*cis* geometry. Only partial resolution of these isomers was achieved by CCD. The amount of *trans*-isomer relative to the all-*cis*-compound decreased with increasing tube number. As expected, the *trans,cis,cis*-isomer migrated more rapidly than the all-*cis*-isomer on silver nitrate-impregnated silica plates. Consequently, preparative TLC proved effective in separating the two.

Since only methyl stearate was formed when the mixed triene esters were hydrogenated, the possibilities of chain branching or of a carbon skeleton other than C_{18} were eliminated.

The ozonolysis fragments from the two unusual trienes are consistent only with a structure having double bonds in the 5, 9, and 12 positions of a C_{18} chain. In addition, a small percentage of ordinary methyl linolenate was also found to be present. The percentage of triene calculated from ozonolysis fragments agrees quite well with the actual percentage of triene in the original sample.

The *trans* monoene provided by partial hydrazine reduction of the *trans* triene contained Δ^5-unsaturation, thereby confirming the position of the *trans* double bond in the triene. The corresponding *cis* monoenes contained Δ^9 and Δ^{12} unsaturation.

The NMR spectra of the two trienes were nearly identical; the only difference in their IR spectra was the presence of the 10.3 μ maximum for the *trans*-ester. This spectral evidence indicated that the two compounds are geometrical isomers. The quartet in the NMR spectrum at τ8.3, indicative of Δ^5-unsaturation, is believed to be associated with a methylene group β to a double bond and also β to a carboxyl group (12). Other significant features of the spectrum include the six proton multiplet for the vinyl protons at τ4.64, a 2 proton triplet at τ7.24 for the methylene group α to 2 vinyl groups, a 2 proton triplet at τ7.75 for the methylene group α to the carboxyl group, an 8 proton multiplet at τ7.93 for protons α to 1 double bond, a six proton multiplet at τ8.70 for shielded methylene groups, a 3 proton triplet at τ9.11 for the terminal methyl group, and the sharp singlet at τ6.39 for the methoxy group. These features agree with the structures for these compounds that were deduced by IR and ozonolysis.

A trace amount of an ester with ECL 18.6 (R-446) was isolated. On the basis of its IR spectrum and ozonolysis results, it seems fairly certain that this ester is methyl *cis*-5,*cis*-9-octadecadienoate.

The natural occurrence of fatty acids with isolated Δ^5 double bonds has been discussed in some of our earlier papers (12,13). Although such acids have been found in a wide variety of sources, none have been encountered previously as a constituent of a seed oil in the plant family Labiatae.

ACKNOWLEDGMENTS

NMR spectra by C. A. Glass and W. A. Boyd; seeds furnished by Q. Jones, Crops Research Division, USDA, Beltsville, Md.

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[Received January 29, 1969]

Molecular Species of Phosphatidyl Ethanolamine From Egg Yolk[1]

B. J. HOLUB and **A. KUKSIS**, Department of Biochemistry and the Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada

ABSTRACT

Phosphatidyl ethanolamine was isolated from total egg yolk lipids by preparative thin layer chromatography (TLC). The purified phosphatide contained 3% of the alkoxy derivative. It was degraded to diglycerides in the presence of purified sphingomyelin by phospholipase C from *Clostridium welchii*. The diglycerides were acetylated and resolved on the basis of unsaturation by argentation TLC. The fatty acid composition of the original phosphatidyl ethanolamine and the derived acetates was determined by gas chromatography, as was the molecular weight distribution of the diglyceride acetates. The placement of the fatty acids in the parent phosphatide was deduced by hydrolysis with phospholipase A from *Crotalus atrox*, and in the acetates with pancreatic lipase. Some 33 major species of phosphatidyl ethanolamine were identified and compared to those for egg yolk lecithins.

INTRODUCTION

Recent studies have indicated that each molecular species of phospholipid must be regarded as having its own metabolic fate. The original work of Collins (1) has been confirmed and extended by others (2,3). Lands and Hart (4) have suggested that this heterogeneity may be due to the enzymes involved in phospholipid metabolism which vary in their specificity towards substrates with different degrees of unsaturation of component fatty acids. This interest has been reflected in the appearance of numerous papers reporting the composition (5,6) and turnover (2-5) of the molecular species of lecithins from various sources. Only sparse information, however, is available regarding the structural and metabolic heterogeneity of the other glycerophosphatides from which standard preparations of phospholipase C do not readily release the diglycerides needed for identification of molecular species.

Quantitative release of diglycerides from phosphatidyl ethanolamine in the presence of 2-acyl glyceryl phosphoryl choline has been reported by Takahashi and Schmid (7) as has been the hydrolysis of bacterial phosphatidyl ethanolamine by phospholipase C from *Bacillus cereus* (8). Neither the latter enzyme nor the 2-lysolecithin, however, are readily available. The present work reports the identification of the major molecular species of phosphatidyl ethanolamine of egg yolk made possible by the successful conversion of this phosphatide into diglycerides by phospholipase C from *Clostridium welchii* in the presence of sphingomyelin, as first suggested by Renkonen (9).

MATERIALS AND METHODS

The methods and general experimental conditions were similar to those previously described (10), except as noted below. All solvent evaporations and enzymic and chemical transformations were performed under nitrogen. A few crystals of purified hydroquinone (British Drug Houses Laboratory Reagent) were added as an antioxidant during extraction and storage of lipids. Chromatographically pure bovine brain sphingomyelin was purchased from General Biochemicals, Chagrin Falls, Ohio, and was further purified by thin layer chromatography (TLC) before use. To check on possible residual contamination with lecithin, a large sample (5 mg) of the sphingomyelin was digested with phospholipase C and the digestion products examined by TLC in petroleum ether-diethyl ether-formic acid (60:40:1.5 v/v). The absence of lecithins was indicated by a failure to detect any diglycerides in the digestion mixture.

Preparation of Egg Yolk Phosphatidyl Ethanolamine

Total lipid extracts were prepared from fresh eggs of White Leghorn hens as previously described (10). The phosphatidyl ethanolamines were isolated from the total lipid extract by preparative TLC on silica gel G (Merck and Co.) using 0.3 mm thick layer spread on 20 X 20 cm plates (De Saga Equipment Co.). About 30 mg of total lipid were applied across the lower edge of each plate. The plates were developed in chloroform-methanol-water (65:25:4 v/v) one or more times as required to effect complete resolution of the lecithins and cephalins. The plates were sprayed with a solution of 0.05% 2,7-dichlorofluorescein in 50% aqueous methanol and the lipid bands located by

[1]Presented in part at the Canadian Federation of Biological Societies Meeting, Kingston, June 1968.

TABLE I

Fatty Acid Composition of Egg Yolk Phosphatidyl Ethanolamines

Fatty	Original			Reconstituted[d]			Diglycerides
	Total[a]	1[b] (mole %)	2[c]	Total	1 (mole %)	2	Total (mole %)
16:0	16.7	32.2	1.4	16.3	32.0	0.7	17.9
16:1	0.5	Trace	0.4	0.4	0.2	0.6	0.3
18:0	27.1	59.2	1.0	29.3	58.2	0.5	28.0
18:1	16.3	7.4	24.9	16.6	8.4	24.9	17.1
18:2	11.2	0.8	22.2	10.5	0.8	20.1	10.4
20:0	0.2	0.1	---	0.1	0.1	---	0.2
20:1	0.3	0.3	0.2	0.2	0.3	0.1	0.2
20:2	0.4	---	0.2	0.3	---	0.6	0.3
20:3	0.4	---	0.8	0.3	---	0.7	0.3
20:4	15.8	---	29.0	17.6	---	35.2	15.9
22:3	1.1	---	1.6	0.5	---	1.0	1.1
22:4	3.1	---	5.7	2.3	---	4.5	2.9
22:5	0.3	---	0.5	0.3	---	0.5	0.4
22:6	6.6	---	12.1	5.3	---	10.6	5.0

[a]Fatty acids of original egg yolk phosphatidyl ethanolamines.

[b]Fatty acids of lyso phosphatidyl ethanolamine after phospholipase A hydrolysis.

[c]Fatty acids liberated by phospholipase A.

[d]Reconstituted values of calculated molecular species from each class of diglyceride acetates.

viewing the plate under ultraviolet light. The purity and identity of the phosphatidyl ethanolamine isolated in this manner was confirmed by TLC with chloroform-methanol-acetic acid-water (25:15:4:2 v/v) and by specific staining (11).

Determination of Alkoxy Phosphatides

Alkoxy derivatives of phosphatidyl ethanolamine were determined by a modification of the method of Horrocks and Ansell (12). The chloroform-soluble phospholipids remaining after acid hydrolysis of the phosphatidyl ethanolamine were isolated by TLC. The lipid fraction, identified as 1-alkyl 2-acyl phosphatidyl ethanolamine, represented 3% of the original phosphorus. This material also was hydrolyzed by phospholipase C, but on TLC (13) the derived 1-alkyl 2-acyl glyceride acetates migrated sufficiently ahead of the 1,2-diacyl glyceride acetates to avoid contamination.

The alkenyl acyl types of phosphatidyl ethanolamine were not determined in this study since previous workers (14,15) had failed to detect such derivatives in egg yolk.

Hydrolysis With Phospholipase C

Free diglycerides were released from the phosphatidyl ethanolamines by incubation with phospholipase C (α-toxin of *Clostridium welchii*) in the presence of sphingomyelin under the conditions developed in our laboratory (Holub and Kuksis, in preparation). The phosphatide (5 mg) was dissolved in 25 ml of ethyl ether containing 1% water along with 1 mg of sphingomyelin. To the mixture were added 20 ml of an autodigested enzyme solution containing 2.5 mg of the phospholipase, 0.35 mmoles of tris-hydroxymethyl aminoethane, 0.02 mmoles $CaCl_2$ adjusted at pH 7.3. The digestion was allowed to proceed at room temperature for 3 hr at which time the reaction mixture was extracted with ethyl ether (3 X 30 ml). The pooled extracts were dried over anhydrous sodium sulfate and the solvent removed under nitrogen. The residue was dissolved in chloroform and the diglycerides isolated by TLC and acetylated as previously described (6,10). The yield of diglyceride acetates after purification by TLC was a minimum of 90-95 mmoles per cent of the starting phosphatide.

Resolution of Diglycerides

The diglyceride acetates were resolved on the basis of total number and distribution of double bonds per molecule by argentation TLC (6,10). The acetates corresponding to the various groups of unsaturation were separately recovered by eluting the gel scrapings with diethyl ether-methanol-acetic acid (60:40:1 v/v) and the subsequent extraction with petroleum. The ratios of the diglyceride acetates among and within the various bands were deter-

TABLE II

Major Diglyceride Acetates of Egg Yolk Phosphatidyl Ethanolamines

Chemical classes	Carbon number	Species as mole % of class	Class as mole % of total mixture
Saturates	---	---	---
Monoenes	36	50.4	24.2
	38	49.6	
Dienes I	36	8.2	2.6
	38	91.8	
Dienes II	36	33.4	18.7
	38	65.2	
	40	1.4	
Trienes	36	3.0	2.0
	38	92.0	
	40	5.0	
Tetraenes I	38	15.3	34.8
	40	81.8	
	42	2.9	
Tetraenes II	40	59.6	3.8
	42	40.4	
Pentaenes	40	70.9	3.1
	42	29.1	
Hexaenes I	40	59.5	10.6
	42	40.5	
Hexaenes II	42	100.0	0.2
Original Mixture	36	17.1	
	38	34.3	
	40	41.5	
	42	7.1	
Reconstituted mixture	36	18.7	
	38	33.7	
	40	39.6	
	42	8.0	

mined by gas chromatography (6,10) with tridecanoin (50-300 μg) as an internal standard.

Analysis of Fatty Acids

The fatty acids were quantitatively estimated by gas liquid chromatography (GLC) following methylation or transmethylation (6,10). The identities of the unsaturated acids were confirmed by argentation TLC and hydrogenation. The fatty acids in the 2 position of phosphatidyl ethanolamine were specifically released by hydrolysis with phospholipase A (*Crotalus atrox*) as described for lecithins (10), except that the incubation time was extended to 4 hr. GLC analysis of the original and the released fatty acids in the presence of internal standard established that a minimum of 88-90 moles per cent of the acid in the 2 position was liberated. The positional distribution of the fatty acids in the diglyceride acetates was determined by hydrolysis with pancreatic lipase (10). In all cases the reaction products as well as any unreacted starting materials were purified and recovered by TLC, prior to the gas chromatographic analysis of the fatty acids.

Calculations

The structure of the original phosphatidyl ethanolamines was calculated by proportional summation and normalization of all the analytical data, as described for egg yolk lecithin (10). The accuracy of the final result was estimated by matching the mole percentage composition, and positional distribution of the fatty acids in the original phosphatidyl ethanolamines, against that determined for the derived diglyceride acetates. In order to obtain a quantitative measure of the degree of specificity noted in the association of saturated and unsaturated acids in these phosphatides, either from similar or different sources, a calculation was devised which permits a comparison between phosphatides of different fatty acid composition. Accordingly, the degree of preference of association was estimated by dividing the ratio of a pair of fatty acids in a particular position of a specific class of phosphatides by the ratio of the same pair of acids in the total mixture. A degree of preference equal to unity represents random association.

RESULTS AND DISCUSSION

The average amount of the phosphatidyl ethanolamine isolated (60 mg/g of total egg yolk lipid) was of the order reported by Noble and Moore (16). Detailed analyses were attempted on the phosphatidyl ethanolamines from three eggs from different hens, but because of accidental peroxidation of some of the polyunsaturated species complete reconstitutions were obtained for only two of them. As far as the analyses were completed, however, there was good agreement among all samples.

Fatty Acid Composition

Table I gives the composition and positional distribution of the fatty acids in one of the preparations of phosphatidyl ethanolamine. The total composition is close to that recorded by Noble and Moore (16), while the positional distribution of the acids is in agreement with that noted by Dyatlovitskaya et al. (17). Their finding of a large proportion of saturated fatty acids in the 2 position of some of the species could not be confirmed in the present study. Slight discrepancies can be attributed to differences in the diets of the birds, which have been shown to affect the composition of egg yolk phosphatidyl ethanolamine (18). The positional distribution of the acids fits well with the established pattern for glycerophosphatides.

Palmitic and stearic are the major acids in the 1 position, while all the polyunsaturated fatty acids are esterified in the 2 position.

Table I also compares the total fatty acids of the original phosphatidyl ethanolamine to those of the derived diglycerides and to those of the diglyceride acetates reconstituted after silver nitrate fractionation. The good agreement between them suggests that both the enzymatic hydrolyses and the subsequent transformations and fractionations were not accompanied by significant selective losses of any molecular species. Slight losses of the species containing docosahexaenoic acid may be attributed to autoxidation of polyunsaturated fatty acid during TLC.

Molecular Weight Distribution

Gas chromatography of the total diglyceride acetates derived from the phosphatidyl ethanolamines of the three egg yolks gave remarkably similar molecular weight distributions. Closely similar elution patterns were also observed for the diglyceride acetates of corresponding degree of unsaturation. On the basis of the fatty acid composition and the molecular weights of the diglycerides it was concluded that the major phosphatidyl ethanolamines in all yolks are those with one C_{16} and one C_{18} (C_{36}), with two C_{18} (C_{38}), and with one C_{18} and one C_{20} (C_{40}) fatty acid per molecule. The slight differences in the proportional contributions of the phosphatides of different molecular weights reflect differences in the fatty acid composition. Table II gives the molecular weight distribution of the total phosphatide and the various classes of unsaturation as estimated by gas chromatography of the derived diglyceride acetates. The lower half of this table includes the reconstitution values obtained for the diglyceride acetates by algebraic summation and normalization of the data from argentation TLC. In Table II the tetraene II + pentaene and the hexaene bands have been numerically resolved on the basis of their fatty acid composition into tetraenes II, pentaenes, hexaenes I and hexaenes II.

On the basis of GLC of the diglycerides we knew that the tetraenes II + pentaenes were made up of carbon numbers 40 and 42. From the fatty acid composition of this fraction, as recovered from our $AgNO_3$ TLC system, we knew that the diglyceride acetates were made up of species containing either 4 or 5 double bonds each. The actual fatty acid pairs were derived by trial and error. The numerical manipulations may be best followed by reference to the data in Table III. Thus the combined tetraene II-pentaenes band contained 11% 20:3

TABLE III

Corrected Fatty Acid Composition of Diglyceride Acetates of Various Degrees of Unsaturation

Fatty acids	Diglyceride acetates[a], mole %								
	Mononeses	Dienes I	Dienes II	Trienes	Tetraenes I	Tetraenes II	Pentaenes	Hexaenes I	Hexaenes II
16:0	25.4		17.2		7.7	29.8		29.8	
16:1	0.8	4.1		1.5	40.9		1.9		
18:0	24.3		32.8			20.2	6.0	20.2	
18:1	48.5	95.9		47.2	1.4		30.1		25.0
18:2			48.8	47.5			11.0		25.0
20:0	0.3			1.3			1.0		
20:1	0.7			2.5					
20:2			1.2				11.0		
20:3					48.6		23.4		
20:4					1.4				
22:3						50.0	9.6		25.0
22:4							6.0		25.0
22:5								50.0	
22:6									

[a]Diglyceride acetates with a total of 0 to 6 double bonds.

TABLE IV

Major Phosphatidyl Ethanolamines of Egg Yolk

Chemical classes	Fatty acids[a] 1	2	Individual species (mole %)	
Saturates			Nil	
Monoenes	16:0	18:1	47.4	
	18:0	18:1	45.6	
	18:0	16:1	1.0	
	18:1	16:0	2.0	24.2
	18:1	18:0	2.0	
	16:0	20:1	0.6	
	20:1	16:0	0.8	
	20:0	16:1	0.6	
Dienes I	18:1	16:1	8.2	2.6
	18:1	18:1	91.8	
Dienes II	16:0	18:2	33.4	
	18:0	18:2	64.1	18.7
	16:0	20:2	1.1	
	18:0	20:2	1.4	
Trienes	16:1	18:2	3.0	
	18:1	18:2	92.0	2.0
	18:1	20:2	2.4	
	20:1	20:2	2.6	
Tetraenes I	16:0	20:4	15.3	
	18:0	20:4	81.8	34.8
	18:1	22:3	2.9	
Tetraenes II	16:0	22:4	59.6	3.8
	18:0	22:4	40.4	
Pentaenes	16:1	22:4	3.9	
	18:1	20:4	45.0	
	18:2	20:3	22.0	3.1
	18:0	22:5	12.0	
	18:1	22:4	15.2	
	20:1	20:4	1.9	
Hexaenes I	16:0	22:6	59.5	10.6
	18:0	22:6	40.5	
Hexaenes II	18:1	22:5	50.0	0.2
	18:2	22:4	50.0	

[a]Fatty acid distribution established by phospholipase A hydrolysis of the original phosphatidyl ethanolamines and by specific lipase hydrolysis of the monoenoic and dienoic diglyceride acetates. Fatty acids and diglyceride acetates identified as in Tables I, II and III.

which corresponded to the 11% of 18:2 giving a pentaene with carbon number 40. Then 6% 22:5 was assumed to have been combined with 6% 18:0 to give a pentaene of carbon number 42. Since the combinations of 20:4 with 16:0 or 18:0 were clearly resolved, any 20:4 in this fraction was assumed to have been derived from combination with 18:1 to give a pentaene of carbon number 40. The 22:4 acid, therefore, occurred in combination with 16:0, 18:0 and any 16:1 and 18:1 left over. When the acids were combined as outlined above, the proportions of fatty acids and diglycerides used gave nearly perfect reconstitutions. The hexaenes I and hexaenes II were similarly resolved.

TABLE V

Association of Palmitic and Stearic Acids With Unsaturated Fatty Acids in the Phosphatides From Egg Yolk

Chemical classes	Major acids in 2 position	Degree of preference[a] for palmitic acid PE	PC
Monoenes	18:1	1.7	1.6
Dienes II	18:2	0.8	0.7
Tetraenes I	20:4	0.3	0.3
Tetraenes II	22:4	2.4	
Hexaenes	22:6	2.4	0.5

[a]Degree of Preference, (16:0/18:0) class per (16:0/18:0) total. Non-preferential association, 1.0 ± 0.1 (assuming a relative error of ± 10%).

The corrected fatty acid compositions of the diglyceride acetates of various degrees of unsaturation are given in Table III. As a result, the contributions of the individual polyenes can now be recognized along with those of the other major classes. A nearly identical distribution was obtained for the second yolk.

The present estimates for monoenes (24.2%) and dienes (21.3%) are considerably higher while those for the hexaenes (10.8%) are lower than the values given by Renkonen (15), who had succeeded in the partial separation of the dinitrophenyl derivatives of the phosphatidyl ethanolamines of egg yolk. The tetraenes (38.6%), surprisingly, showed good agreement. These discrepancies may be due to differences in the diets of the birds, since the fatty acid composition of the egg yolk phosphatides given by Renkonen (15) deviated greatly from the values determined in this study as well as from those found by others (16,17).

Molecular Species

Table IV lists the major individual phosphatidyl ethanolamines of egg yolk. The estimates were derived by reconstitution of the overall molecular weight distribution (Table II) and the fatty acid composition (Tables I and III). The algebraic methods used in these calculations and the manner of specifying the molecular species have been described (10).

An examination of Table IV shows a remarkable specificity in the association of the fatty acids in the phosphatide molecules. Thus, there are no detectable amounts of fully saturated species, although the proportion of saturated fatty acids in these phospholipids is considerable (44.0%). The major monoenes are (16:0 16:1) and (18:0 18:1) phosphatidyl ethanolamines, occurring in nearly equal proportions. Since there is much less palmitic than stearic acid in the original mixture, it represents

a preferential association of palmitic and oleic acids in these phosphatides. This observation is in agreement with that of Arvidson (3), who noted a comparable preference for this type of pairing in rat liver phosphatidyl ethanolamines. The major dienes are the (16:0 18:2) and (18:0 18:2) species. In this case the species containing stearic acid was present in double the amount of the species containing palmitic acid, as would have been expected on a random basis. The tetraenoic fraction containing arachidonic acid had considerably more stearic than palmitic acid, while the reverse was true for the tetraenoic fraction incorporating docosatetraenoic acid. In both cases the experimental distributions deviated significantly from those anticipated on a random basis. Interestingly, the ratio of the hexaene species containing palmitic and stearic acids was identical to that found for the tetraenes containing docosatetraenoic acid. In both cases the long chain fatty acids showed a marked preference for palmitic acid.

Comparable specificities in the association of the saturated and unsaturated fatty acids have been observed for the monoenoic and the dienoic fractions by Dyatlovitskaya et al. (17), and polyenoic species of the phosphatidyl ethanolamines of egg yolk by Renkonen (15). Hill et al. (19) and Arvidson (3) also noted a strong preferential association of docosahexaenoic and palmitic acids in the phosphatidyl ethanolamines of mammalian liver. Dyatlovitskaya et al. (17) failed to quantitate the polyenoic species in their egg yolk phosphatidyl ethanolamines.

Previous workers failed to note the marked difference in the preference for saturated fatty acids in the two tetraene types. Apparently the selection of palmitic over stearic acid in the long chain polyenes is based on differences in molecular weight rather than degree of unsaturation. In contrast to the earlier noted differences between the present estimates and those of Renkonen (15) for the total mononenes, dienes and hexaenes, it may be observed that there are practically no qualitative differences in the species at least as far as comparable analyses have been completed. This is in agreement with the data obtained for the molecular species of phosphatidyl cholines isolated from different yolks (10). The dietary differences are manifested more prominently in the proportions of the different classes of unsaturation of the phosphatides than in their qualitative make-up.

Comparison With Egg Yolk Lecithins

In view of the uncertainty of the exact biosynthetic origin of the glycerophosphatides, it was deemed desirable to compare the molecular species of the phosphatidyl ethanolamines and cholines of egg yolk. The comparison was made on the basis of both the nature of the fatty acid involved and the degree of preference shown. For the calculation of the degree of preference, a characteristic ratio of proportions was set up as illustrated in Table V, which also gives the results of some of the associations thus measured. The original values for the egg yolk lecithins were taken from an earlier study from this laboratory (10). In the monoenes, dienes II and tetraenes I of both phosphatidyl ethanolamine and phosphatidyl choline, the major unsaturated acids preferred the same saturated fatty acid in position 1. Furthermore, in all cases the values calculated for the actual degree of preference were in very close agreement. There was, however, a marked difference exhibited in the kind of fatty acid found in the 1 position of the hexaenes in the two phosphatides. While the phosphatidyl ethanolamine contained mainly palmitic acid, the phosphatidyl cholines contained mostly stearic acid. In view of the apparent equilibration of the tetraenes it is possible that the 1-stearoyl, 2-arachidonoyl lecithin of egg yolk could have originated from the corresponding phosphatidyl ethanolamine, as already claimed by Balint et al. (2) for the rat liver lecithins. Without having dynamic data available on the transformations and turnover of these phospholipids, further comments regarding the significance of the specificity in the fatty acid association seem unwarranted.

ACKNOWLEDGMENT

These studies were supported by grants from the Ontario Heart Foundation, Toronto, Ontario, and the Medical Research Council of Canada. The National Research Council of Canada provided a fellowship to B. J. Holub.

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[Received January 29, 1969]

Glyceride Structure of *Erlangea tomentosa* Seed Oil, A New Source of Vernolic Acid

B. E. PHILLIPS,[1] C. R. SMITH, JR. and J. W. HAGEMANN,
Northern Regional Research Laboratory,[2] Peoria, Illinois 61604

ABSTRACT

(+)-Methyl vernolate constitutes 52% of the methyl esters formed by transmethylating the oil of *Erlangea tomentosa* seed. Thin layer chromatography fractionated the oil into trivernolin, divernolins, monovernolins and nonvernolate glycerides. Pancreatic lipolyses of the monovernolins and the divernolins followed by transmethylation of the monoglycerides indicated the presence of 34 vernolate glycerides above the 0.1% level, grouped as follows: α-monovernolins, 15%; β-monovernolins, 3%; α,α'-divernolins, 10%; α,β-divernolins, 37%; trivernolin, 16%; and nonvernolate glycerides, 19%.

INTRODUCTION

The search for economical sources of oils containing appreciable amounts of epoxides is stimulated by their potential usefulness as plasticizers in commercial products, such as poly(vinyl chloride), and as additives to protective and decorative coatings. Further, epoxyacyl groups may have biosynthetic significance as intermediates in some plant seeds as Gunstone (1) has suggested. In his review Krewson (2) lists four long chain, epoxy acids that have been found in seed oils. These compounds have been isolated from the seed of more than 40 species of 12 plant families (1,3). Further studies (4,5), including unpublished results from this Laboratory, indicate that small quantities (less than 5%) of epoxy acids occur in many seed oils. So far *Vernonia anthelmintica* seed oil has yielded the largest percentage of an epoxy acid; i.e., 70% or more of the acids from the oil were *cis*-12,13-epoxy-*cis*-9-octadecenoic (6).

Contemporary methods of separation coupled with enzymatic procedures have permitted the rapid determination of the types of glycerides present in epoxide seed oils (8,9). Tallent et al. (8) have reported the trivernolin, divernolin and monovernolin levels in three seed oils, and Fioriti et al. (10), the vernoloyl distribution of recrystallized *V. anthelmintica* seed oil. Further Tallent et al. (8) were able to estimate the amounts of α- and β-monovernolins and α,α'- and α,β-divernolins by enzymatic methods. When the screening program of this Laboratory showed that the seed of *Erlangea tomentosa* S. Moore, a native of tropical Africa (7), contained an appreciable quantity of one or more epoxy acids, the epoxy-containing compound was isolated, identified and its glyceride distribution ascertained.

EXPERIMENTAL PROCEDURES

Chromatographic Separations

Thin layer chromatography (TLC) analyses and separations were carried out on glass plates spread with 0.25 and 1.0 mm layers of Silica Gel G in the following solvents: Solvent A, petroleum ether-ethyl ether (80:20); Solvent B, petroleum ether-ethyl ether (65:35); Solvent C, hexane-ethyl ether (1:1); and Solvent D, hexane-ethyl ether-acetic acid (50:50:1). Analytical plates were visualized by charring at 120 C with the sulfuric acid-chromic oxide reagent. The bands on the preparative plates were located under an ultraviolet (UV) lamp after spraying with dichlorofluorescein or by a visual examination on a light table if concentrations were high enough. Samples were washed from the silica gel with ethyl ether or Solvent C. Approximately 85% of the methyl esters and 55% of the partial glycerides were recovered from the preparative chromatograms. Epoxy compounds were located with the picric acid spray described by Fioriti and Sims (11). Methyl esters were tentatively identified by measuring the equivalent chain lengths according to the procedures developed by Miwa et al. (12) on a Resoflex 446 or an Apiezon L column or both. Relative amounts of the various reaction products were estimated by measuring the peak areas of the respective gas liquid chromatography (GLC) analyses with an Infotronic integrator. Peak areas were assumed proportional to the masses of the respective compounds and were converted to mole percentages. The partial glycerides were examined chromatographically on an OV-1 column by methods similar to those described by Tallent and Kleiman (9).

Isolation of Seed Oil

Ground seed of *E. tomentosa* (3.0 g) were extracted with petroleum ether in a Soxhlet extractor. The resulting solution was concentrated

[1]Postdoctoral Research Associate 1968-1969.
[2]No. Utiliz. Res. Dev. Div., ARS, USDA.

TABLE I

Erlangea tomentosa Seed Oil Fractionation

R_f	Recovered sample, mg[a]	Nature of compounds in fractions
0.87	7.0 (1.3%)	Unidentified
0.81	62.7 (11.4%)	Nonepoxy glycerides (C_3)
0.73	55.6 (10.1%)	Monovernolins (C_2V)
0.64	196.3 (35.6%)	Divernolins (CV_2)
0.55	65.8 (12.0%)	Trivernolin (V_3)
0.43-0.00	163.6 (29.7%)	Free fatty acids (FA)

[a]The combined mass of these fractions represents 77% of the material placed on the plate.

under nitrogen to yield 0.71 g of yellow oil. Infrared (IR) absorption at 822 and 844 cm^{-1} suggested the presence of an epoxide. Titration with the Durbetaki reagent (hydrogen bromide in glacial acetic acid) at 55 C (13) indicated that the epoxyacyl group (calculated as vernolate) accounted for 52% of the seed oil.

Characterization of the Epoxy Acid

A 0.209 g portion of the seed oil was acetolyzed as described by Gunstone (14). The resulting acids were esterified in refluxing acidic methanol (1% sulfuric acid). By TLC in Solvent D, the esters were separated into three groups of compounds: nonoxygenated esters (32%), an unidentified group of esters of intermediate R_f (16%) and the dihydroxy methyl esters (52%). A 35.6 mg portion of the dihydroxy esters (1.08×10^{-4} mole as methyl dihydroxyoleate) in ethanol absorbed 1.05×10^{-4} mole of hydrogen under 1 atm of hydrogen with platinum as catalyst. The reduction product, when crystallized from hexane, had a mp of 67-68 C. Gunstone (14) reported a mp of 67.5-69 C for methyl 12,13-dihydroxystearate. The hydroxystearate (30 mg) was oxidized with permanganate-periodate according to von Rudloff's procedure (15). Before GLC analyses on the Apiezon and Resoflex columns, a portion of the acids was methylated with acidic methanol. Hexanoic acid and methyl dodecanedioate accounted for nearly all the short chain acids and diesters. A similar oxidation of a 30 mg portion of the unreduced dihydroxy esters yielded hexanoic and nonanedioic acids.

Sodium methoxide transmethylation of the seed oil and subsequent TLC of the esters in Solvent A yielded essentially pure methyl epoxyoleate. The methyl vernolate thus isolated migrated homogeneously in all four solvent systems and on both GLC columns. The specific rotation and the optical rotatory dispersion curve, $[\alpha]^{26}_{589}$ +2.07° (c 1.82, hexane), $[\alpha]_{400}$ +3.17°; $[\alpha]_{350}$ +2.91°, $[\alpha]_{300}$ +0.91°, $[\alpha]_{280}$ -1.16°, agree with those reported by Powell et al. (16) for (+)-methyl vernolate. The IR spectrum of the ester, both as a liquid film and in carbon tetrachloride (10%), did not have any appreciable absorption at 968 cm^{-1} (*trans*-monoene) but did absorb at 822 and 845 cm^{-1} (epoxide).

Glyceride Composition of the Oil

A 0.71 g portion of the seed oil was fractionated into eight distinct groups of compounds by preparative TLC in Solvent B, and the fractions were washed from the adsorbent with ethyl ether. The amounts of each fraction obtained are listed in Table I. Table II gives the GLC analyses of each fraction after transmethylation with 0.2 M sodium methoxide.

Lipolysis of the Monovernolins

The pancreatic lipase (E.C. 3.1.1.3) hydrolyses of the mono- and divernolins were patterned after the procedure of Luddy et al. (17) which was modified slightly. A mixture of 1.0 ml of 1 M Tris buffer (pH 8.0), 0.1 ml of 22% calcium chloride, 0.25 ml of 0.1% sodium cholate and 0.020 g of steapsin (Nutritional Biochemicals Corp., Cleveland, Ohio), were equilibrated at 40 ± 1 C for several minutes. The lipase was washed twice with acetone, twice with ether, and then dried over calcium sulfate in vacuo before use. After the monovernolins, 44 mg (49 μmole), were added, the mixture was vigorously agitated with a Vortex mixer for 4 min at 40 C. The suspension was diluted with 20 ml of cold ether and 0.5 ml of 6 N sulfuric acid. The fatty acids and monoglycerides were separated from the aqueous phase by ether extraction, and the acids were methylated with ethereal diazomethane. Preparative TLC in Solvent C divided the reaction mixture into four fractions. GLC analyses of each of the groups of compounds indicated the following products. The nonepoxy methyl esters, 11.0 mg (mass adjusted to represent only the amount derived from monovernolins), equivalent to 37 μmole of linoleic acid, liberated during the lipolysis were: $C_{14:0}$, 0.1%; $C_{15:0}$, 0.1%; $C_{16:0}$, 13.9%; $C_{18:0}$, 14.5%; $C_{18:1}$, 23.8%; $C_{18:2}$, 46.0%; $C_{18:3}$, 0.3%; $C_{20:0}$, 0.8%; $C_{20:1}$, 0.2%. This ester sample contained 0.2% methyl vernolate. The methyl vernolate fraction, 8.7 mg (28 μmole), was isolated in the amount expected if glyceryl β-vernolates constituted 15% of the monovernolins. The third fraction contained small amounts (ca. 1 mg) of diglycerides and triglycerides. The slowest migrating fraction, 9 mg, was comprised principally of monoglycerides. GLC analysis on the OV-1 column indicated that vernolate esters made up 11% of the mono-

TABLE II

GLC Analyses of Methyl Esters Derived From Glyceride Fractions[a]

Acyl group	C_3[b]	C_2V		CV_2		V_3[d]	FA[d]	Original oil[d]
		Whole[b]	β position[c,e]	Whole[d]	β position[b,e]			
C-14:0	<0.1	---	---	---	0.6	---	0.1	<0.1
C-15:0	<0.1	0.1	0.2	---	0.6	0.1	0.1	<0.1
C-16:0	11.0	6.0	1.1	4.2	1.9	0.4	5.8	5.0
C-18:0	10.5	6.3	1.3	3.8	0.9	0.3	3.9	4.8
C-18:1	29.6	18.5	22.7	7.6	4.4	0.8	10.9	11.4
C-18:2	46.4	41.6	58.0	24.3	12.1	3.7	27.9	26.1
C-18:3	0.7	0.2	0.9	---	---	---	0.1	0.1
C-18:1E[f]	0.4	27.0	15.5	60.2	79.4	94.6	51.0	52.0
C-20:0	1.2	0.3	0.2	---	---	---	0.1	0.2
C-20:1	0.2	0.1	0.2	---	---	---	---	0.1

[a]The structural assignments are tentative and are based on GLC elution times. The relative amounts were determined by integrating peak areas and converting to mole per cent.

[b]Averages of GLC analyses on the Resoflex 446 and Apiezon L columns.

[c]Values are based on GLC analyses on the OV-1, Resoflex 446, and Apiezon L columns.

[d]Resoflex 446 column only.

[e]After transmethylation of monoglycerides.

[f]Methyl vernolate.

glycerides. Sodium methoxide transmethylation of the monoglycerides and GLC analyses of the derived methyl esters yielded the data given in Table II.

Lipolysis of Divernolins

Similarly, 142 mg (156 μmole) of divernolins was lipolyzed at 40 C with 40 mg of pancreatic lipase suspended in 3 ml of 1 M Tris buffer, 0.3 ml of 22% calcium chloride and 0.75 ml of 0.1 M sodium cholate for 4 min. Agitation was provided by a rapidly spinning Teflon-coated bar magnet in a capped vial. The reaction products were recovered in a manner analogous to that used for the monovernolins, and they were similarly separated into five major fractions. The nonepoxy methyl esters, 18 mg (equivalent to 62 μmole of methyl linoleate), were comprised of the following: $C_{15:0}$, 0.3%; $C_{16:0}$, 12.1%; $C_{18:0}$, 14.1%; $C_{18:1}$, 18.3%; $C_{18:2}$, 54.0%; $C_{18:3}$, 0.5%; $C_{20:0}$, 0.3%; $C_{20:1}$, 0.3%. Methyl vernolate, 33 mg (106 μmole), constituted 62% of the second major TLC fraction according to GLC analysis on the OV-1 column. The third major TLC fraction contained 24 μmole of normal triglycerides and 5 μmole of trivernolin (presumably synthesized by the lipase (18) since the original divernolins appeared to be free of trivernolin). The diglycerides, 15 mg (24 μmole), which migrated ahead of the monoglycerides, were found by GLC analysis on the OV-1 column (after silylation) to be a mixture of monovernoloyl and divernoloyl diglycerides. The ratio of mono- and divernoloyl diglycerides, 1.53:1.00, is that expected from the divernolins, if they are 80% α(α'),β-divernolins. The recovered monoglycerides, 15 mg, were found to be principally glyceryl vernolate. The groups bound to the β positions of the divernolins are listed in Table II.

DISCUSSION

Approximately half of the methyl esters obtained by sodium methoxide transmethylation of the seed oil of *E. tomentosa* was (+)-methyl vernolate. This ester was shown to be methyl (12*S*,13*R*)-*cis*-12,13-epoxy-*cis*-9-octadecenoate by the following: The von Rudloff oxidation of the acid-hydrolyzed ester yielded principally hexanoic and nonanedioic acids. A similar oxidation of the reduced dihydroxy ester, a dihydroxystearate obtained by acetolysis and hydrogenation of the seed oil, yielded hexanoic and dodecanedioic acids. The lack of any significant IR absorption at 968 cm^{-1} indicated the one double bond was of *cis* configuration. The ORD curve of the methyl vernolate is similar to that of the methyl vernolate found in *V. anthelmintica* seed oil, which has a *cis*-epoxy group with a 12*S*,13*R*-configuration (17,20). Thus the epoxy group of the vernolate in *E. tomentosa* seed undoubtedly has the same configuration.

Although separation of the seed oil into homogeneous classes of compounds was not completely successful, TLC analyses of the more rapidly migrating fractions, in conjunction with GLC analyses of derived methyl esters, indicated the following mole ratio of triglycerides in *E. tomentosa* seed: nonepoxy

TABLE III

Principal Glycerides in *Erlangea tomentosa* Seed Oil

Glyceride substituents[a]			Total of each fraction	Nonepoxyacyl groups[b]							
α	β	α		15	16	18	18:1	18:2	18:3	20	20:1
V	V	V	16.0								
V	A	V	9.7	---	0.9	0.5	2.1	5.6	---	---	---
V	V	A	37.0	---	3.6	3.8	6.5	21.8	---	---	---
V	A		14.7	T	0.2	0.2	4.0	10.1	0.1	T	T
V	18:1	A	4.0	---	0.5	0.5	0.9	2.2	---	---	---
V	18:2	A	10.1	---	1.2	1.3	2.2	5.5	---	---	---
A	V	A	2.9								
A	V	A		---	T	T	0.1	0.9	---	---	---
16	V	A		---		0.1	0.1	0.4	---	---	---
18	V	A		---			0.2	0.4	---	---	---
18:1	V	A		---				0.7	---	---	---
A	A	A	19.8								

[a]V, vernoloyl group; A, substituents listed under the acyl groups. No distinction has been made between the 1 and 3 positions of the glycerol moieties and both diastereomers are listed together.

[b]Numbers are mole per cent of specified triglycerides in the seed oil. All other estimates are rounded off to the nearest 0.1 mole per cent. ---, Indicate these components are not present above the 0.1% level. Blank spaces indicate the isomer is listed elsewhere in the table. T shows those groups definitely present at levels less than 0.1%.

glycerides, 20%; monovernolins, 18%; divernolins, 47%; and trivernolin, 16%. More polar glycerides such as would have resulted from the opening of an epoxide were not considered since they migrated with the fatty acids.

Even though it has been demonstrated that not all straight chain acids are cleaved at the same rate (18,20) and that vernoloyl groups are hydrolyzed more slowly than oleoyl groups (21), we have assumed that the monoglycerides produced by the pancreatic lipase fairly represent the acyl groups bound to the β positions of the original glycerides. The divernolins present in the seed oil, and listed in Table III, were determined directly from the ester ratios shown in Table II. The ratios of substituents attached to the α positions of the monvernolate glycerides were estimated by evenly distributing the acyl groups not bound at the β positions among the available α positions of both the α- and β-vernolates.

E. tomentosa is another member of the family Compositae which produces seeds with appreciable quantities of vernolates. The manner in which the epoxyacyl groups are distributed among the different triglycerides is far from random with approximately five of six monovernolins as α-vernolates and four of five divernolins as α(α′),β-divernolates. This pattern is in marked contrast to the picture one might have obtained by lipolysis of the unfractionated glycerides since 35% of all the β positions of the triglycerides are esterified to vernolic acid.

The glyceride ratio of *E. tomentosa* seed oil has only a limited similarity to the glyceride ratios reported by Tallent et al. (8) and Fioriti et al. (10) even though two of the oils they studied are from plants (*Crepis aurea* and *V. anthelmintica*) that are members of the family Compositae. The ratios of the α-monovernolins to the β-monovernolins in *E. tomentosa*, *Euphorbia lagascae* (8), and *V. anthelmintica* (10) are each four or five to one. Only *E. tomentosa* and *V. anthelmintica* (11) have similar proportions of divernolins; i.e., 70% to 80% of the divernolins are α(α′),β-divernolins. However, the amounts of the divernolins and trivernolin in the *V. anthelmintica* oil differ markedly from that of *E. tomentosa* (10). In overall composition the seed oil of *E. tomentosa* is most like that reported for *E. lagascae* (8), i.e., 10% nonepoxy glycerides, 3% β-monovernolins, 12% α-monovernolins, 52% α(α′),β-divernolins, 4% α,α′-divernolins, and 19% trivernolin. Vernolate makes up 58% of the glycerol esters of this oil.

ACKNOWLEDGMENT

E. tomentosa seed supplied by Robert Perdue, New Crops Research Branch, ARS, USDA. R. G. Powell measured the ORD of the methyl vernolate.

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[Received April 28, 1969]

Bio-Oxidation of Linoleic Acid via Methylmalonyl-CoA[1,2]

JACQUELINE DUPONT and MELVIN M. MATHIAS[3], U. S. Army Medical Research and Nutritional Laboratory, Fitzsimons General Hospital, Denver, Colorado 80240

ABSTRACT

Unsaturated long-chain fatty acids are oxidized more rapidly than are saturated fatty acids of similar chain length in intact animals and isolated mitochondria. Gamma-oxidation of the 3-dodecenoic acid intermediate in beta-oxidation of oleate would yield propionate which is metabolized via methylmalonate. ^{14}C-labeled fatty acids were administered to intact rats, muscle homogenates and lysed mitochondria. Methylmalonate, succinate and CO_2 were isolated and ^{14}C determined. Incorporation of U-^{14}C-linoleate into methylmalonate in vitro was 20 times greater than from U-^{14}C-palmitate. Rats fed 20% corn oil grew more slowly on B_{12} deficient than B_{12} sufficient diets. Biotin and vitamin B_{12} deficiencies were found to decrease the in vivo metabolism of linoleate. These data suggest that one pathway of linoleate oxidation has methylmalonate as an intermediate.

INTRODUCTION

In 1956 Mead et al. (1) reported a study of fatty acid oxidation in mice. Fatty acids labeled with ^{14}C were fed and expired CO_2 was collected. The total ^{14}C expired in 24 hr from stearate was 29.1% of the dose; from oleate, 57.8% and from linoleate, 38.4%. In a series of studies, Goransson and Olivecrona (2a) and Goransson (2b,c,d) compared disappearance of an intravenous dose of ^{3}H-palmitate with other fatty acids labeled with ^{14}C in rats. They found that in 320 hr oleic, palmitoleic and linoleic were more rapidly lost than palmitic. Stearic acid was lost more slowly than palmitic. Lynn and Brown (3) reported that ^{14}C-linoleate was oxidized more rapidly than ^{14}C-stearate when they were fed to rats. Dupont (4) has also reported that linoleate was oxidized 10 times as fast as stearate when the fatty acids were injected into rats intraperitoneally.

Lynn and Brown (3) have demonstrated that lysed rat mitochondria oxidize carboxyl-labeled linoleate and linolenate to CO_2 more rapidly than they oxidize stearate. Bressler and Friedberg (5), using beef heart mitochondria, found that linoleate and oleate were oxidized more rapidly than palmitate. More recently Bjorntorp (6) compared oxidation rates of albumin-bound fatty acids C2 through C18, and mono- and di-unsaturated C18 fatty acids. Incubation with rat liver mitochondria yielded oxidation rates which followed Michaelis-Menten kinetics. Short chain fatty acids were oxidized more rapidly tnan longer chains on a molecular basis. When calculated on a carbon basis shorter chains were also oxidized more rapidly, except for linoleate, which was oxidized as rapidly as butyrate.

Unsaturated fatty acids of C16 and C18 were more rapidly oxidized than saturated fatty acids of the same length in all the studies cited. There are several sites in the overall absorption and metabolism of fatty acids where differences in rates may exist. The facts that isolated mitochondria behave like the intact organism and that route of administration of the fatty acids has no effect suggest that one of the rate differences exists at the mitochondrial level of metabolism. Lynn and Brown (3) attributed their findings to a greater activity of activating enzymes for the unsaturated fatty acids. Bjorntorp (6) concluded from his studies that adsorption of the fatty acid to the mitochondrion did not account for the difference in rates of oxidation.

Complete oxidation of fatty acids has long been assumed to be via β-oxidation. β-Oxidation cannot account for complete oxidation of unsaturated fatty acids. The double bond in the 9 position makes total β-oxidation impossible unless the double bond is shifted to an even position. Stoffel (7) has proposed a pathway for oxidation of unsaturated fatty acids by which the double bond shifts from the 9 to the 8 position. The existence of this pathway has been clearly demonstrated, but it does not necessarily account for more rapid oxidation of unsaturated than saturated fatty acids.

Sinclair (8) proposed that hydration may occur at the γ double bond which would be present after 6 carbons had been removed by β-oxidation. The result would be the splitting off of a propionyl-CoA molecule. This pathway could account for the more rapid oxidation of

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[1]Presented in part at the 12th International Conference on the Biochemistry of Lipids, Loughborough, England, September 1968.

[2]Scientific Series Paper No. 1384, Colorado Agricultural Experiment Station.

[3]Present Address: Department of Food Science and Nutrition, Colorado State University, Fort Collins 80521.

TABLE I

Composition of Diets

	Experiment 1[a] g/100g	Experiment 2[b], g/100g		
		Beef tallow	Corn oil	Propionate
Protein	30.0[c]	20.4[d]	20.4	20.4
Salt mix	4.0[e]	4.0[f]	4.0	4.0
Carbohydrate	54.7[g]	49.9[h]	49.9	47.9
Cellulose	4.0	5.0	5.0	5.0
Corn oil[i]	7.3	0	20.0	20.0
Beef tallow[j]	---	20.0	0	0
Na-propionate	---	0	0	2.0
Vitamin mix	+[k]	0.7[l]	0.7	0.7

[a]Conducted at Colorado State University.

[b]Conducted at U.S. Army Medical Research and Nutrition Laboratory.

[c]Vitamin-free casein was replaced with desiccated eggwhite in the biotin deficient group, General Biochemicals, Chagrin Falls, Ohio.

[d]Vitamin-free casein supplemented with 4 g of L-methionine per kilogram of diet, Nutritional Biochemicals, Cleveland, Ohio.

[e]Jones and Foster, GBI.

[f]Rogers and Harper, NBC.

[g]Cornstarch, GBI.

[h]Dextrin, NBC.

[i]Mazola, obtained on local retail market.

[j]Rendered beef kidney fat.

[k]Milligram per kilogram of diet: thiamine-HCl, pyridoxine-HCl, riboflavin, 5.0; niacin, Ca-pantothenate, 20; folic acid, 2.0; B_{12}, 0.03; menadione, 4.0; calciferol, 0.1; vitamin A acetate, 3.0; α-tocopherol, 100; para-aminobenzoic acid, inositol, 200; choline Cl, 2,000. Controls received 0.2 mg of biotin per kilogram of diet.

[l]Modified from Leveille et al. (16) to contain per kilogram of diet: vitamin A, 10,000 IU; vitamin D_2, 2,000 IU; (in mg) α-tocopheryl acetate, 100; menadione, 0.05; biotin, 0.44; Ca-pantothenate, 66.0; folic acid, 2.0; *p*-aminobenzoic acid, 110.0; inositol, 110.0; niacin, 100.0; pyridoxine-HCl, 22.0; riboflavin, 22.0; thiamine-HCl, 22.0; L-ascorbic acid, 1,000; and choline Cl 3,000. Controls received 0.1 mg crystalline vitamin B_{12} per kilogram of diet.

unsaturated than saturated fatty acids.

The metabolism of propionic acid has been reviewed by Kaziro and Ochoa (9). The series of reactions leading into the citrate cycle is:

$$\text{Propionyl-CoA} + HCO_3^- + ATP \xrightarrow[\text{carboxylase}]{\text{Biotin-dependent}} \text{Methylmalonyl-CoA} + ADP + P_i$$

$$\text{Methylmalonyl-CoA} \xrightarrow[\text{mutase}]{B_{12}\text{-dependent}} \text{Succinyl-CoA}$$

This pathway leads to the synthesis of oxaloacetate which may then be available for priming the citrate cycle without participation of carbohydrate. This could logically account for more rapid oxidation of unsaturated fatty acids. Another interesting aspect of this proposed pathway is the possibility of synthesis of carbohydrate from unsaturated fatty acids by the classical gluconeogenic pathway.

The present experiments have been conducted to determine whether unsaturated long chain fatty acids yield methylmalonate as Sinclair proposed.

MATERIALS AND METHODS

Sources of dietary ingredients are shown in Table I. Radioactive fatty acids were obtained from Applied Science Laboratories, State College, Pennsylvania, or Tracerlab, Waltham, Massachusetts. The non-radioactive fatty acids were obtained from the Hormel Institute or Applied Science. Collection of CO_2, in vivo, was as previously reported (10). The in vitro incubations (11) were carried out in 25 ml Erlenmeyer flasks stoppered with rubber serum stoppers which were fitted with a hanging plastic cup. At the end of the incubation, 0.2 ml ethanolamine-methylcellosolve (1:2 v/v) was injected into the cup using a needle and syringe and the incubation was stopped and the CO_2 liberated by injecting 0.5 ml of 0.6N $HClO_4$ directly into the medium. The incubation was continued for at least 90 min to allow absorption of the CO_2. The cup and its contents were counted in 15 ml of Bray's scintillant (12).

Extraction of dicarboxylic acids from liver was by homogenization in a Waring Blendor with 5 vol of acetone acidified with HCl. The

TABLE II

R_f Values on Silica Gel[a] Developed With a Water-Saturated Diethyl Ether-Formic Acid (15:1) Solvent[b]

Acid	R_f
Malic	0.58
Lactic	0.67
β-hydroxybutyric	0.73
Succinic	0.82
Methylmalonic	0.87
Fumaric	0.90
Fatty Acids	1.00

[a]Mallinckrodt precoated plates 7 G.
[b]See reference 24.

homogenate was filtered and solvents evaporated. The residue was dissolved in a small amount of acid acetone and an aliquot used for thin layer chromatography.

Incubation flask contents were adjusted to pH 12 by addition of NaOH to hydrolyze any methylmalonyl-CoA present (13,14). Carrier dicarboxylic acids were added. The sample was then acidified to pH 2 and homogenized in a Waring Blendor with 2 vol of acetone. The homogenate was filtered and the acetone evaporated. The aqueous residue was extracted 3 times with 2 vol of ethyl ether. The ethyl ether was evaporated and the residue treated the same as liver extracts. Organic acids were extracted from urine by washing 3 times with ethyl ether after acidification to pH 2 with HCl.

Thin layer chromatography of dicarboxylic acids was performed on pre-coated silica gel plates, obtained from Mallinckrodt, by the method of Ting and Dugger (15). The plates were activated at 100 C for 30 min prior to use. Samples were streaked on 5 x 20 cm plates. The developing solvent used by Ting and Dugger was water-saturated diethyl ether and 88% formic acid in a ratio of 7 to 1. For the separation of methylmalonate from fumarate we found a ratio of ether-formic acid 15:1 to be satisfactory. Plates were developed for approximately 65 min at room temperature in solvent saturated glass tanks. Bands were detected by spraying with a mixture of 0.1% methyl red and 0.3% brom phenol blue (1:1). The bands were yellow to orange on a blue-purple background. Table II shows the R_f values of some organic acids as determined in this laboratory. Absolute R_f varied from time to time, but relative position of the acids found was constant. Bands were scraped from the plates into scintillation vials and counted in toluene scintillant containing 4 g 2,5-diphenyloxazole and 50 mg 1,4-di 2-(5-phenyloxazolyl)-benzene/liter. Radioactivity determinations were made using a Beckman Liquid Scintillation system Model LS200. Corrections were made for background counting and quenching.

EXPERIMENTAL PROCEDURES

In Vivo Experiments

Experiment 1. Young adult female rats (Sprague-Dawley derived, Laboratory Suppliers, Atlanta) were made deficient in biotin by feeding a 30% egg white diet without biotin (Table I) for 11 weeks. These and the controls fed casein and biotin were given a dose of U-^{14}C-palmitate or -linoleate as the sodium salt intraperitoneally. Expired CO_2 was collected for 1 hr and the rats were then killed and liver methylmalonate extracted and analyzed.

Experiment 2. Male Holtzman rats (50-60 g) were fed diets containing different fat sources at the level of 20% with or without supplemental vitamin B_{12} (Table I). Corn oil was substituted for beef tallow in order to change the dietary intake of linoleate. As a further stress of the vitamin B_{12}-requiring systems 2% sodium propionate was added to the corn oil diet (17-20).

After the rats were on their respective

TABLE III

Incorporation of U-^{14}C-Palmitate or -Linoleate Into Methylmalonate and CO_2 by Female Rats[a]

Label	Biotin	^{14}C expired in 1 hr[b] Per cent of dose	^{14}C in methylmalonate[c] dpm/liver
Palmitate	+	2.60 ± 0.25	60 ± 31
Palmitate	-	3.82 ± 0.43	10 ± 3
Linoleate	+	7.82 ± 1.64	290 ± 226
Linoleate	-	6.22 ± 1.08	23 ± 9

[a]Total of 0.8 μc per rat (630 mc/m mole) as the Na salt.
[b]Six rats per group.
[c]Three to four rats per group.

TABLE IV

Body Weight at 6 Weeks After Weaning of Male Holtzman Rats Fed a Diet Containing 20% Fat ± Vitamin B_{12}

Dietary treatment[ab]	Number or rats	Average body weight, g
I Beef tallow - B_{12}	11	258 ± 36[c]
II Beef tallow + B_{12}	10	270 ± 20
III Corn oil - B_{12}	18	234 ± 29
IV Corn oil + B_{12}	17	282 ± 23
V Corn oil + 2% Propionate - B_{12}	7	178 ± 10
VI Corn oil + 2% Propionate + B_{12}	7	260 ± 33

[a]See Table I for detailed composition.

[b]Statistical "t" tests: I vs. II: ns; I vs. III: ns; II vs. IV: ns; III vs. IV: $<.001$; I vs. V: $<.001$; II vs. VI: ns; V vs. VI: $<.001$; III vs. V: $<.001$; IV vs. VI: ns.

[c]Mean ± standard error.

dietary treatments for 6 weeks, U-^{14}C-linoleate was administered intraperitoneally. Urine was collected for 4 days and analyzed for radioactive dicarboxylic acids.

In Vitro Experiments

Whole Homogenate. Rat hearts were homogenized in 9 vol of Krebs phosphate buffer minus $CaCl_2$ and the homogenates were incubated with U-^{14}C-palmitate, -oleate or -linoleate (5) in albumin complex. Methylmalonate and succinate were isolated and ^{14}C determined.

Lysed Mitochondria. Heart, red (*rectus femoris*) and white (*adductor magnus*) skeletal muscles were obtained from male Holtzman rats fed laboratory chow. A 10% homogenate was made in 0.175 M KCl using an Omni-Mixer, then a Teflon-pestle tissue homogenizer. Nuclei and cell debris were removed by centrifuging at 600 X *g* for 10 min; the supernatant was recentrifuged at 8500 X *g* for 20 min at 4 C. The mitochondrial fraction was resuspended in distilled water. The incubation medium was that used by Fritz and Yue (21) minus malate. Protein was determined by the microbiuret procedure.

RESULTS

In Vivo Experiments

Biotin deficient rats incorporated significantly less radioactivity from U-^{14}C-linoleate into methylmalonate than control rats (Table III). As previously shown (1-4), linoleate was oxidized more rapidly than palmitate in control animals ($P < 0.005$). The difference between linoleate oxidation rates in biotin deficient and control rats was not significant. Palmitate oxidation was significantly greater ($P < 0.025$) in biotin deficient than control rats. Biotin deficiency decreased the *in vivo* incorporation of both fatty acids into methylmalonate; the effect on linoleate was more severe.

Vitamin B_{12} deficiency as evaluated by growth retardation, shown in Table IV, was produced by a corn oil basal diet but not by one containing beef tallow as the sole dietary

TABLE V

Urinary Excretion of ^{14}C-Methylmalonate and -Succinate From U-^{14}C-Linoleate of Male Rats Fed 20% Beef Tallow or Corn Oil ± Vitamin B_{12} for 6 Weeks After Weaning[a]

Vitamin B_{12}	Time	Beef tallow, dpm/rat		Corn oil, dpm/rat	
		Succ.	Memal.	Succ.	Memal.
+	0-12	218	603	1281	2379
+	12-24	289	166	244	348
+	24-48	266	214	355	59
-	0-12	344	1275	1243	2223
-	12-24	185	355	229	547
-	24-48	141	207	222	659

[a]Total of 2.9 μc rat (630 mc/m mole) as the Na salt. Three rats per group.

TABLE VI

Incorporation of U-^{14}C-Fatty Acids Into Methylmalonate by Whole Rat Heart Homogenates[a]

Label	Methylmalonate dpm/mg tissue
Palmitate	6
Oleate	16
Linoleate	54

[a]Medium: Krebs phosphate buffer minus $CaCl_2$, 0.014 mM bovine serum albumin, 0.1 mM fatty acid (2 mc/m mole), 11 mg tissue per flask. Total volume, 1.2 ml. Incubated 30 min at 37 C.

fat ($P < 0.001$). A further growth depression ($P < 0.001$) was found when 2% sodium propionate was incorporated into the corn oil basal diet deficient in vitamin B_{12}.

Radioactivity found in urine of these rats for the 48 hr following intraperitoneal injection of U-^{14}C-linoleate is shown in Table V. In the first 12 hr after injection of U-^{14}C-linoleate rats fed corn oil with or without vitamin B_{12} supplement excreted more labeled methylmalonate and succinate than rats fed beef tallow, but rats fed beef tallow with vitamin B_{12} excreted the least labeled methylmalonate and succinate of all groups. In the second 12 hr and in the second day there were no differences between beef tallow with or without B_{12} and corn oil with B_{12}. The rats fed corn oil without B_{12} continued to excrete ^{14}C-methylmalonate through the second day, while those receiving B_{12} excreted very little ^{14}C-methylmalonate on the second day.

In Vitro Experiments

Incorporation of U-^{14}C fatty acids into methylmalonate by whole heart homogenates is shown in Table VI. The incorporation of label into methylmalonate follows the same pattern as production of $^{14}CO_2$ from ^{14}C-fatty acids as reported by Bressler and Friedberg (5).

Lysed mitochondria from different muscle tissues were incubated with U-^{14}C-linoleate or -palmitate. The data in Table VII indicate that ^{14}C-methylmalonate and -succinate produced from linoleate was 20 to 30 times that from palmitate in heart and white muscle mitochondria. The different results in palmitate metabolism by red skeletal muscle are of interest to the study of energy metabolism.

DISCUSSION

Some indirect evidence has accumulated to suggest that linoleate can be catabolized by the metabolic pathway which exists for propionate. Hahn et al. (22) have consistently shown an increase in blood glucose level after linoleate

TABLE VII

Incorporation of U-^{14}C-Palmitate or -Linoleate Into CO_2, Succinate and Methylmalonate by Lysed Mitochondria From Muscles of Male Rats Fed Stock Diet[a]

	Palmitate, dpm/mg protein			Linoleate[b], dpm/mg protein		
	CO_2	Succinate	Methylmalonate	CO_2	Succinate	Methylmalonate
Heart	2048 ± 394[c]	488 ± 136	392 ± 179	1343 ± 177	6778 ± 2641	7846 ± 3219
Red muscle	537 ± 103	1335 ± 356	1902 ± 509	607 ± 90	6475 ± 2258	5054 ± 2305
White muscle	337 ± 83	325 ± 93	394 ± 208	507 ± 139	9967 ± 5035	9645 ± 5163

[a]Concentration of medium (in mM): fatty acid, 0.1 (1.6 mc/m mole); bovine serum albumin (fatty acid poor), 0.014; sucrose, 100; EDTA-Na, 0.4; KCl, 80; Na phosphate buffer (pH 7.4), 8; $MgCl_2$ 5; ATP-Na, 1; NAD-Na, 2.5; coenzyme A, 0.04; dl-carnitine, 1; 1-5 mg mitochondrial protein. Total volume 2.5 ml. Incubated 30 min at 37 C.

[b]$NaHCO_3$, 25 mM, added to medium.

[c]Mean ± standard error, 5-7 incubations.

intubation to infant rats. The finding that saturated fats are more ketogenic than unsaturated fats (10,23,24) further supports the premise that linoleate has the potential to supply oxaloacetate.

We have demonstrated that biotin and vitamin B_{12} deficiencies adversely affected in vivo linoleate utilization. The incorporation of U-^{14}C-linoleate into urinary ^{14}C-methylmalonate and the slower rate of its disappearance from the urine of vitamin B_{12}-deficient rats (Table V) indicated that linoleate was a source of urinary methylmalonate. It could be one of the sources of urinary methylmalonate excreted by vitamin B_{12} deficient humans (25,26), rats (27,28) and hamsters (29). The ability of a diet containing highly unsaturated lipid (Table IV) to enhance a vitamin B_{12} deficiency resembles the well-known effect of dietary propionate (17-20). The slight growth retardation caused by a deficiency of vitamin B_{12} in the group receiving beef tallow was probably caused by the oleate in beef tallow.

These results strongly support Sinclair's hypothesis that unsaturated fatty acids can be partially oxidized via the pathway which exists for propionate catabolism. The physiological significance for this pathway and the one proposed by Stoffel (7) needs to be studied. Recent reports have suggested that complete oxidation of oleate and linoleate occurs in liver only via β-oxidation (30,31). Anderson (30) suggests that U-^{14}C-linoleate yields primarily acetoacetate and β-hydroxybutyrate upon incubation with isolated rat liver mitochondria. Antony and Landau (31) give evidence that 10-^{14}C oleate yields predominantely acetate upon incubation with liver slices in the presence of glucose.

Our studies have shown that oleate is metabolized at a different rate from linoleate, but that both yield greater amounts of methylmalonate in vivo and in vitro than palmitate. Liver metabolizes fatty acids differently from muscle. Whether all possible pathways exist in all tissues or different tissues use specific pathways remains to be determined.

The primary question to be answered has been by what means unsaturated fatty acids are more rapidly oxidized than saturated fatty acids. Proof of the methylmalonate pathway will require demonstration that methylmalonate is derived directly from unsaturated fatty acids and not from saturated ones. The presently reported studies indicate strongly that such is the case, and that in consequence the oxidation of unsaturated fatty acids is more rapid than that of saturated fatty acids.

ACKNOWLEDGMENTS

Supported in part by the Colorado Heart Association and by Western Regional Research Funds (Project W-91). Mitochondrial experiments, G. R. Beecher; technical assistance, J. Taubr, M. Block, N. Romeo; animal care, B. James (U. S. Army Medical Research and Nutrition Laboratory).

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[Received January 6, 1969]

Preparation of ^{14}C-Labeled Fatty and Anacardic Acids from *Ginkgo biloba*

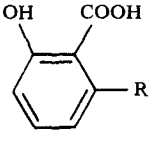
JOANNE L. GELLERMAN and **HERMANN SCHLENK,**
University of Minnesota, The Hormel Institute, Austin, Minnesota 55912

ABSTRACT

Acetate labeled with ^{14}C was infused into shoots of Ginkgo. Up to 16% of the radioactivity was incorporated into lipids. Most of it was found in the leaves and stems, but after 9 days, the lipids of roots also contained an appreciable amount of radioactivity. Radioactive palmitic, linoleic and other common acids were isolated as methyl esters; 5,11,14-20:3 and esters of similar type were isolated pure or together with their isomers of normal structure; anacardic acids were separated as such from the fatty esters.

INTRODUCTION

An investigation of the toxic effects of anacardic acids (6-alkylsalicyclic acids) on microorganisms has been undertaken in this laboratory (1) on the basis of recent advances in analysis and preparation of these acids (2).



R is n-alkyl, C_{13}, C_{15}, or C_{17} with 0 to 3 double bonds; abbreviated An13:0, An15:1, etc.

Anacardic acids labeled with ^{14}C were needed for further pursuit of such studies and it was decided to prepare them by biological synthesis from ginkgo plants. Ginkgo produces An15:1 and An17:1 as major anacardic acids. In addition, 5,11,14-20:3 and related polyenoic acids with a double bond remote from others occur in ginkgo lipids together with common, saturated and unsaturated fatty acids (3,4).

In view of the simplicity of application, the use of radioactive acetate as precursor seemed preferable to $^{14}CO_2$. The technique involves infusion of the aqueous solution of the radioactive precursor by means of a wick which is threaded through the stem of the plant. This procedure had been used mainly with small amounts of precursors at low level radioactivity to explore biosynthetic pathways (5). Therefore some pilot experiments were made to ascertain the efficiency of this method for our preparative purposes. These results are reported together with those of a larger scale experiment where 6 mc 1-^{14}C-acetate was applied.

PROCEDURES AND RESULTS

Ginkgo plants were used outdoors with shoots of one season's growth from four year old roots. The biggest diameter of the stems was about ¼ in. and the plants were 23 to 30 in. in height when the radioactive sodium acetate was administered in experiments during August 1968. Heavy cotton knitting yarn was threaded through the stem just above the first leaf from the ground. One end of yarn was cut flush with the stem and the other end was cut to 3-4 cm in length. A 5 ml beaker was mounted somewhat below that point, but not fastened to the stem. The end of the yarn was placed into the beaker and sodium acetate, 2 mg (200 μc ^{14}C) or 82 mg (2,000 μc ^{14}C) in 2 ml water was added. The solution was taken up in daylight within 4-12 hr varying with individual plants and weather conditions. At least 2 ml of distilled water was then added to rinse residual acetate from the beaker and wick into the plant. Time periods from beginning of infusion to harvesting were 1½ to 9 days as specified in Table I.

Leaves, stems and, with the higher activity preparation, also the roots were cut, weighed and extracted twice in an Omnimixer with $CHCl_3$:CH_3OH, (2:1 v/v). The extracts were washed with 0.2 volume of 0.9% NaCl in water. Aliquots of the chloroform phases were taken to dryness, weighed and counted in a scintillation counter. The extracts were colored and correction factors for quenching were determined from model mixtures with radioactive standards. The main portions of lipids were saponified by refluxing for 4 hr in 2N ethanolic KOH. Nonsaponifiables were extracted with diethyl ether.

Fatty and anacardic acids were recovered and the former were esterified selectively by reflux for 30 min in 10 vol of dry methanol containing 3% sulfuric acid (2). The mixture of fatty esters and anacardic acids was extracted by diethyl ether after addition of 10 vol of water. Esters and acids were then separated by chromatography on silicic acid. For example, 70 g SiO_2 (Mallinckrodt 100 mesh) was used in a column 3 cm O.D. and 30 cm long for 1.6 g of the mixture. The adsorbent had been acti-

vated at 120 C overnight but was then slightly deactivated to minimize trailing of anacardic acids. Commercial hexane with diethyl ether, increasing in concentration from 1% to 4% was used as developing solvent and eluent (2). The effluent fractions were combined according to radioactivity and TLC analysis of aliquots.

The specific activity of anacardic acids and fatty acid methyl esters was determined (Table I). The anacardic acids consisted mainly of An15:1 and An17:1 and further fractionation was not necessary for our purpose. The distribution of radioactivity among individual anacardic acids was determined on microscale by GLC of their dimethyl ether esters. GLC was carried out in aluminum tubing, 107 cm x 6.3 mm O.D., packed with cycloheptaamylose acetate, 5% on Chromosorb W, silicone treated, 60-80 mesh (Johns-Manville), at 236 C and 3.1 atm inlet pressure (2). The fractions were collected and counted (6) (Table II).

The purity of anacardic acids from preparation 3, leaves plus stems, was carefully checked. About 3.5% of the radioactivity appeared in GLC with the solvent front (Table II) whereas their chemical purity was about 99.8%. Special experiments showed that the contaminants were mainly fatty acids which emerge as methyl esters close to the solvent front in GLC of anacardic acid dimethyl ether esters. The ratio of specific activities of fatty acids-anacardic acid is about 20:1 (Table I). Therefore, these small amounts of fatty acids account for the relatively large percentage of radioactive impurity in this area. The selective esterification procedure was repeated by refluxing the sample for 20 min in 1.33N HCl in dry methanol. After chromatography on SiO_2 96% of the anacardic acids were recovered. Their radioactive contamination had been decreased from 3.5% to 0.2%. Accordingly, their chemical contamination by fatty acids is in the order of 0.01%.

Fatty esters obtained by the initial separation from anacardic acids were analyzed by GLC and counting (6) (Table II). Individual fatty esters from Experiment 3, leaves plus stems, were then isolated by liquid-liquid chromatography followed by GLC of portions up to 200 mg (4,6). Their specific activities were determined and are listed in Table III.

DISCUSSION

More than 700 mg of anacardic acids having a ^{14}C activity of approximately 9 x 10^4 DPM/mg have been obtained in high purity. Between 4 and 15 mg of polyenoic fatty acids with a double bond structure 5,11... have been

TABLE I

Incorporation of ^{14}C From Sodium Acetate Into Ginkgo Lipids

No.	Experiments: Na-acetate ^{14}C in	Na-acetate μc	Na-acetate mg	Time, days	Plant harvested, g	mg Lipid extract	Yields: Crude Lipid % ^{14}C	Unsaponifiable mg	Unsaponifiable % ^{14}C	Fatty acids mg	Fatty acids % ^{14}C	Fatty acids DPM/mg	Anac. acids mg	Anac. acids %^{14}C	Anac. acids DPM/mg
1[a]	C-1	200	2.04	1.5	26[b]	653	8.8	71	0.55	112	8.0	317,100	105	0.22	9,570
2[a]	C-2	200	2.24	7	29[b]	612	15.9	91	1.60	136	12.7	413,500	138	0.85	27,410
3[c]	C-1	6,000	246	9	132[b]	3,365	9.03	595	0.72	594	7.2	1,607,300	766	0.51	88,250
					168[d]	1,320	0.91	262	0.04	302	0.64	280,800	154	0.01	7,650

[a]One plant.
[b]Leaves + stem.
[c]Three plants.
[d]Roots.

TABLE II

Percentage Composition and Activity of Acids[a]

Exp. No.	Composition, %		Radioactivity, %			
	3		1	2	3	
	Leaves + stems	Roots	Leaves + stems	Leaves + stems	Leaves + stems	Roots
Anacardic Acids						
Solvent front	---	---	2.9	1.6	3.5[b]	18.4
An13:0	3.2	3.6	6.6	5.1	9.1	3.0
An15:1	60.9	74.2	70.0	65.8	73.6	57.9
An17:1	35.9	21.6	20.5	27.4	18.7	20.7
Fatty Acids[c]						
14:0	3.2	---	1	1.2	1.6	---
16:0	22.1	17.2	31.4	28.8	23.9	26.9
16:1	3.6	2.9	2.4	2.7	2.6	2.5
18:0	4.5	---	3.2	3.4	2.7	---
18:1	9.2	12.9	33.7	21.6	17.0	31.3
18:2	14.1	32.8	19.2	25.5	33.1	25.8
18:3	37.5	10.0	3.8	10.8	10.7	3.6
20:3	3.1	14.4	3.4	4.2	5.2	5.0
20:4	1.4	---	0.4	0.7	1.2	---

[a]GLC area per cent and per cent ^{14}C in collected fractions of anacardic ether esters and fatty esters.

[b]Mainly fatty acid methyl esters.

[c]Structures, see Table III.

isolated and their activity was in the order of 10^6 DPM/mg. In addition, numerous common fatty acids of similar activity were obtained. The specific activities of individual compounds from the same biosynthetic experiment are different (6) and one has also to expect different levels of activities within each compound (7). Unequal labeling may be particularly pronounced in molecules of anacardic acids where an aromatic ring and an aliphatic chain are combined.

When comparing specific activities, it is seen that ^{14}C was incorporated more efficiently from 2-^{14}C- than from 1-^{14}C- acetate (Table I, Exp. 2 vs. 1 and 3). Incorporation in fatty acids was essentially the same after 1½ and 9 days whereas the proportion of radioactivity in anacardic acids had increased within this period

TABLE III

Fatty Acid Methyl Esters Isolated From Leaves and Stem of Ginkgo[a]

Compound	mg	DPM x 10^6/mg	Position of double bonds[b]
14:0	6.9	1.07	
16:0	106.6	1.69	
16:1	9.7	1.30	9- and *trans*-3-[c]
16:2	2.2	0.95	7,10- and 9,12-
16:3	15.2	0.53	mainly 7,10,13-
18:0	4.6	1.70	
18:1	51.0	2.39	mainly 9-
18:2	66.7	3.14	87% 9,12-; 13% 5,11- or 5,9-
18:3	166.1	0.36	9,12,15- with 1% 5,11,14-
20:1	0.7	2.41	?
20:2	4.6	0.94	5,11-
20:2	2.2	2.03	11,14-
20:3	15.6	1.59	5,11,14-
20:4	10.2	0.22	5,11,14,17-

[a]Experiment 3 of Tables I and II.

[b]By comparison of retention times with those of authentic samples (4).

[c]Compound newly reported from ginkgo leaves; identified by ozonolysis, IR spectrum and mp.

(Exp. 1 and 3). The biosynthetic sequences leading to anacardic acids apparently proceed slower than the synthesis of fatty acids.

Although the percentage of radioactivity incorporated in fatty acids did not markedly change after 1½ days, the distribution of ^{14}C shifted with time in favor of the more unsaturated acids. For example, chemical percentages of 18:2 in leaves and stem are similar in experiments 1, 2 and 3 while its radioactive percentage was higher in the longer term experiments 2 and 3. The distribution of ^{14}C among fatty acids in roots after 9 days is very similar to that of the fatty acids in the leaves from the shorter term experiment 1. Similarly, the percentage of ^{14}C in anacardic acids is smaller in leaves from experiment 1 and in roots from experiment 3 than in leaves from experiments 2 and 3. Under the assumption that transport of lipids as such does not take place in the plant, one may state that with the acetate infusion method the radioactive syntheses are slower in roots than in leaves.

The radioactive synthesis of fatty acids is of more general interest than that of anacardic acids and other plants than ginkgo can be used for synthesis of the former. Between 8% and 16% ^{14}C was recovered from acetate as lipids and between 7% and 13% ^{14}C was in fatty acids. Puncture and infusion of an aqueous solution of 4% sodium acetate did not cause any visible damage to the small ginkgo plants. Similarly, one can expect that the tolerance to radiation damage permits higher dosage than 2 mc of ^{14}C per plant as it had been used here.

Radioactive lipids often have been prepared from bacteria or algae and acetate, but plants usually are less demanding in maintenance than microorganisms. With plants, in many cases the wick method and ^{14}C acetate will be more expedient than photoassimilation of $^{14}CO_2$ since infusion is less complicated in regard to apparatus and precautions (8,9). Radioactive yields by infusion may be lower than by other methods (9), but no special equipment is required.

ACKNOWLEDGMENTS

This investigation was supported in part by PHS research grants no. AM 05165 and HE 08214 from the National Institutes of Health, Public Health Service and by The Hormel Foundation. The authors thank E. Leete for helpful discussion and Miss N. Walsh for technical assistance.

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[Received January 3, 1969]

A Comparative Study of Gangliosides From the Brains of Various Species

C. -L. SCHENGRUND[1] and O. W. GARRIGAN, Department of Chemistry, Seton Hall University, South Orange, New Jersey 07079

ABSTRACT

A comparison is made of the sialic acid and hexose content of crude gangliosides from the brains of 13 species, including mammals, a bird, an amphibian, a reptile and fish. The sialic acid content is relatively constant from species to species. Gangliosides are less abundant in tissues other than the brain. Four species (cat, dog, pig and sheep) were selected for the determination of their major ganglioside subfractions. The ganglioside subfractions were isolated (after extraction and partition dialysis) using descending thin layer chromatography. The population of ganglioside molecules varied from species to species. From dog and sheep a mono-, a di- and a trisialoganglioside were obtained; from cat a mono-, a di- and two trisialogangliosides; and from pig a mono- and two disialogangliosides. Each ganglioside subfraction was found to contain glucose, galactose and galactosamine in the ratio of approximately 1:2:1. The fatty acid moieties consisted of more than 80% stearic acid with lesser amounts of arachidic, palmitic and behenic acid. Sphingosine analyses indicated ratios of sphingosine to icosisphingosine of 7:3 for the monosialo-, 1:1 for the disialo- and 3:7 for the trisialogangliosides.

INTRODUCTION

It has been recognized for some time that the term ganglioside includes a variety of molecular species. Among the lipids, gangliosides are uniquely complex; they are theoretically capable of bearing information for species specificity. Patterns of ganglioside variability may correlate with differences in function among the various ganglioside types and divergent pathways of biosynthesis.

A survey of gangliosides was undertaken to find out which of the possible variations are actually realized in a representative selection of brain tissue. It was also of interest to learn if gangliosides prepared on different occasions from the same species varied as much from each other as gangliosides prepared from different species. Ganglioside materials from tissues other than the brain were not studied with any thoroughness.

The gangliosides of several species were examined in greater detail to determine if the composition of ganglioside subfractions is consistent from species to species. A convenient apparatus for the descending thin layer separation of ganglioside subfractions is described. The general composition of the subfractions, their content of sphingosine and icosisphingosine and their fatty acid profile were determined.

EXPERIMENTAL PROCEDURES

Materials

Ox, pig and sheep brains were obtained from commercial abattoirs within an hour of slaughter and packed in ice or dry ice for transport to the laboratory. Mink brains were obtained on the day of slaughter from a commercial mink ranch. Cod (*Gadus morrius*) were obtained in mid-winter at a New York market (caught off the coast of New England). The heads were kept in ice for several hours before the brain tissue was extracted.

The dogs, cats, rabbits and frogs were adult laboratory animals. The dogs and cats had been used (under Nembutal) for blood pressure and urine experiments respectively, in a medical student physiology laboratory; the experiments were terminated by allowing air into the coronary arteries of the animals. The rabbits were made insensitive in an atmosphere of carbon dioxide, and bled rapidly from the throat. The rats (200 g adults) were decapitated. The frogs (*Rana pipiens*) were pithed before the removal of the brains. The Florida alligators (*Alligator mississippienis*) were 1 year old and about 50 cm long; they were killed by rapid freezing. In each case, the brains were removed, frozen if necessary, and the extraction procedure begun within several hours of the animals' death.

The electric eel (*Electrophorus electricus*) brains were obtained from live animals over the course of several days and kept at -10 C until sufficient pooled material was collected.

Separation of Gray Matter From White Matter

Gray matter was separated from the underlying white layer of cerebral cortex tissue of the ox, sheep, pig, dog and cat.

[1] Present address: Department of Biological Chemistry, College of Medicine, Pennsylvania State University, Hershey, Penn. 17033.

It was found that removing the gray matter by suction (water pump) resulted in a more rapid and thorough separation of gray from white than by the most careful dissection. The gray matter was stripped from the tissue and drawn into a sidearm flask through a short piece of glass tubing.

Isolation of the Gangliosides

The gangliosides were extracted from the tissue using the modified partition dialysis method of Folch-Pi et al. (1,2). At first the gangliosides were purified by redistribution in chloroform-methanol solution through a column of cellulose. It was found, however, that the ganglioside subfractions could be isolated by direct thin layer chromatography (TLC) of the crude (unpurified) gangliosides.

The ganglioside subfractions were isolated using preparative descending TLC. An all glass descending TLC apparatus (Fig. 1) was constructed, suggested by the model of Privett et al. (3). A 10 cm X 17 cm plate was spread with silica gel H, prepared according to Stahl, and activated at 130 C for 3 hr (4). A 10 mg portion of the total ganglioside sample to be fractionated was applied to the plate. The solvent used for elution of the sheep ganglioside subfractions was propanol-water (7:3 v/v) (4). The solvent used for elution of the cat, dog and pig subfractions was propanol-water (3:1 v/v) (5). The latter solvent system gave better separation of the ganglioside subfractions. The chamber was saturated with the solvent before the plate was inserted. A Whatman No. 1 paper wick was used to carry the solvent from the solvent trough to the top of the plate. This wick was found to give the best flow rate for separation of the subfractions. The plate was developed for five days. Separate fractions (about 0.5 ml each) were collected every 1.5 hr. The first gangliosides were eluted in about 36 hr. Every fifth fraction was taken for analysis by ascending TLC. The ascending TLC plates (coated with silica gel G) were developed in propanol-water (3:1 v/v) for 5 hr, and the ganglioside spots visualized with resorcinol spray (6). Fractions were combined on the basis of identical TLC behavior for the analysis of their carbohydrate and sphingosine components.

The advantage of eluting the ganglioside subfractions from the plate by descending TLC is that the fractions may be taken directly for analysis. When the fractions are extracted from ascending TLC plates, some silica gel is also extracted. A second purification step is necessary to obtain the ganglioside subfraction alone. Although good separation of the ganglioside subfractions was obtained, a major problem

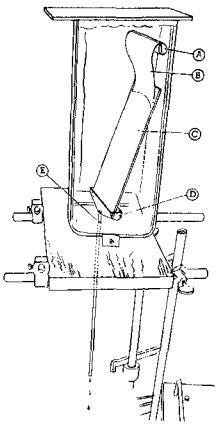

FIG. 1. Preparative descending TLC apparatus for the isolation of ganglioside subfractions. The chamber consists of a battery jar closed at the top, with a hole drilled in the bottom for a tight-fitting tube E leading to the fraction collector. The solvent trough A is supported by a glass frame. B is the paper wick; C the TLC plate and D the collecting trough.

with the descending TLC is the length of time required for complete elution of the different gangliosides from the plate. Sialic acid has been reported to be split off by prolonged contact with silica gel (7). However, no splitting off of sialic acid was observed in the following experiment. Cat ganglioside subfractions eluted from a descending TLC plate were combined and run a second time. The relative amount and composition of each subfraction remained the same. In addition, monitoring by ascending TLC consistently showed the same pattern of ganglioside subfractions both before and after descending TLC.

The ganglioside subfractions taken for fatty acid analysis were isolated using preparative ascending TLC. A 10 X 20 cm plate was spread with silica gel H and activated at 130 C for 3 hr. The plate was developed in propanol-water (3:1 v/v) for 4-5 hr, removed from the chamber and air dried. The plate was scraped in 1 cm sections which were collected separately. A 2 cm strip running the length of the plate was not scraped. The spots on this strip were visualized with resorcinol spray. This method was less time-consuming than the descending TLC; fatty acids were shown to remain intact during exposure to TLC and the contaminating silica gel was tolerable in the analysis of fatty acids.

Methods of Analysis for the Ganglioside Components

Sialic acid was determined using the direct Ehrlich reaction (8). The other carbohydrate moieties (glucose, galactose and N-acetyl-

TABLE I

Specimens of Crude Ganglioside Preparations From Brain Tissue

Specimen	Number of brains pooled	Per cent sialic acid in ganglioside	Ganglioside mg/g wet tissue	Per cent solids in tissue[c]	Hyperphasic sialic acid as per cent dry weight
Electric	10	15.8	2.1	15.0	0.25
eel	6	20.9	2.2		
Cod	30	11.1	4.1	15.9	0.29
	43	11.1	2.4		
Frog	109	20.2	1.1	14.5	0.15
Alligator	10	21.9	3.1	14.0	0.49
Chicken	33	16.2	3.2	17.2	0.34
	61	16.1	3.8		
	51	20.8	2.5		
	229	15.8	3.8		
Mink[b]	19	17.8	3.3	21.6	0.27
Rat	6	21.5	2.0	20.0	0.33
	11	20.4	3.1		
Rabbit	2	15.3	4.2	21.0	0.26
	6	24.8	3.1		
	3	11.1	5.7		
Ox[a]	8	20.8	3.2	20.2	0.33
	10	18.9	3.5		
	10	21.1	2.9		
Pig[a,b]	7	13.4	2.7	17.7	0.21
	78	20.2	2.8		
Sheep[a]	8	15.5	3.7	18.3	0.34
Cat[a]	8	17.9	3.0	19.4	0.28
	5	21.1	2.5		
Dog[a]	11	18.8	4.4	18.6	0.43
	12	15.8	5.3		
	2	18.0	3.9		

[a]Gangliosides were prepared from isolated gray matter.

[b]Unpublished data of E. J. Iorio and O. W. Garrigan, Seton Hall University.

[c]Averaged value for all preparations.

galactosamine) were isolated using the hydrolysis procedure given by Ledeen and Salsman (9). The hydrolysis of the ganglioside subfractions and the subsequent neutralization of the solution by passage through a mixed ion exchange column was accompanied by no detectable loss of hexose. Total hexose was determined both before and after hydrolysis using the anthrone reaction (10). Qualitative identification of the sugars by paper chromatography using *n*-butanol-pyridine-water (9) indicated only glucose and galactose in the hydrolysate. The spots were visualized with an aniline-phthalic acid spray (11) which was found to be sensitive to as little as 10 μg of sugar. Approximately 400 μg of crude gangliosides were used for the isolation of the sugars. Therefore, any sugar present and not detected would account for less than 2% of the ganglioside. When more than 60 μg of either sugar was present in the mixture applied to the paper chromatogram, overloading became noticeable and the separation of glucose from galactose was not clear at the end of the 20 hr developing period. Glucose was determined using the glucose oxidase method (12) and galactose by difference between the glucose and the total hexose content. Hexosamine was analyzed using the Elson-Morgan procedure (13). Galactosamine was qualitatively identified by degradation to pentose with ninhydrin in a sealed capillary tube, followed by chromatography using butanol-ethanol-water (4:1:1 v/v/v) (14). The sphingosine bases were extracted from the hydrolysate used for the carbohydrate analyses. The sphingosine was oxidized to give fatty acids in an extension of the method of Sweeley and Moscatelli (15), as developed by Rosenberg and Stern (16). A permanganate-periodate reagent (17) was used for oxidation at the double bond; 2.0855 g of sodium periodate and 0.3951 g of potassium permanganate were dissolved in 100 ml of water. One milliliter of the permanganate-periodate solution was mixed with 4 ml of redistilled *t*-butanol (A. Rosenberg, private communication). Sphingosine samples of less than 1 mg were dissolved in 1.0 ml of the

TABLE II

Sialic Acid and Hexose Content of Crude Gangliosides Isolated From the Species Studied and of the Individual Subfractions of Cat, Dog, Pig and Sheep Brain Gangliosides

Source	Ganglioside Subfraction	Weight per cent sialic acid	Weight per cent hexose	Molar ratio hexose-sialic acid
Electric eel	Crude	18	14	2:2
Cod	Crude	11	12	3:2
Frog	Crude	20	18	3:2
Alligator	Crude	22	18	3:2
Chicken	Crude	18	22	4:2
Rat	Crude	21	22	3:2
Rabbit	Crude	17	18	3:2
Ox	Crude	20	27	4:2
Pig	Crude	20	23	4:2
	Monosialo-	14	16	1.9:1
	Disialo-$_1$	24	20	2.8:2
	Disialo-$_2$	20	17	2.8:2
Sheep	Crude	17	16	3:2
	Monosialo-	17	14	1.3:1
	Disialo-	29	22	2.5:2
	Trisialo-	33	21	3.0:3
Cat	Crude	20	18	3:2
	Monosialo-	11	11	1.7:1
	Disialo-	22	17	2.6:2
	Trisialo-$_1$	34	21	3.0:3
	Trisialo-$_2$	35	17	2.4:3
Dog	Crude	18	24	4:2
	Monosialo-	14	15	1.8:1
	Disialo-	27	20	2.4:2
	Trisialo-	29	14	2.4:3

reagent and the oxidation was allowed to proceed at room temperature for 4-24 hr. At the end of the time allowed for oxidation, solid sodium hydrosulfite was added, with mixing, until all of the purple permanganate and brown manganese dioxide were reduced. The pale yellow solution was cooled in an ice-water bath and acidified with concentrated hydrochloric acid. The fatty acids obtained from the oxidation were heated with boron trifluoride-methanol (47% w/v and freshly prepared) (18) for 5 min at 100 C to obtain the methyl esters (16).

The fatty acids of the ganglioside subfractions were hydrolyzed and the methyl esters were formed with boron trifluoride-methanol without prior removal of the silica gel. Three hours were sufficient for hydrolysis and methyl ester formation (16).

The fatty acid methyl esters were determined both qualitatively and quantitatively using a Research Specialties Series 600 gas liquid chromatograph. A 6 ft x 1/8 in. stainless steel column, packed with 15% diethylene glycol succinate on chromasorb WAW 60/80 was used for the analysis. The column temperature was 195 C and the flow rate of the nitrogen carrier gas was 45 ml/min. The identification of the fatty acid methyl esters was confirmed using a 6 ft x 1/8 in. stainless steel column, packed with 20% Apiezon L on Chromasorb W. The column temperature was 260 C and the flow rate of the nitrogen carrier gas was 10 ml/min.

The identification of the fatty acids using these columns was confirmed by using hydrogenation and internal standards consisting of known fatty acid methyl esters.

RESULTS

Survey of Brain Gangliosides From Various Species

Crude ganglioside preparations were made from brain tissue of 13 species, including mammals, a bird, an amphibian, a reptile and fish, in quantities listed in Table I. The amount of crude gangliosides and the content of sialic acid in the gangliosides were both quite constant in tissues of such diverse origin. The variation from species to species was of the same order of magnitude as the variation among preparations from the same species. It may be noted that the sialic acid content of gray matter (when the brains were developed enough to make this discrimination practical) did not differ widely from the sialic acid content of whole brains in the lower species. The content of sialic acid in the ganglioside fraction, averaged over

TABLE III

Carbohydrate Content of the Major Ganglioside Subfractions of the Gray Matter of Cat Brain and of the Total Gangliosides of the Gray Matter of Cat, Dog, Pig and Sheep Brains

Ganglioside subfraction	Per cent galactosamine	Per cent galactose	Per cent glucose	Ratio of galactosamine-galactose-glucose
Cat monosialo-	3.0	5.0	3.2	1:1.7:1.1
Disialo-	5.5	9.9	5.5	1:1.8:1.0
Trisialo-$_1$	6.1	12.9	5.1	1:2.1:0.8
Trisialo-$_2$	5.4	9.7	6.3	1:1.8:1.2
Total	3.7	10.0	3.7	1:2.7:1.0
Dog total	5.6	10.0	5.9	1:1.8:1.1
Pig total	4.5	9.7	5.4	1:2.2:1.2
Sheep total	5.0	7.2	4.0	1:1.6:0.8

the 13 species, was 0.31% of the dry weight of tissue.

The hexose to sialic acid ratio found for crude gangliosides is shown in Table II. Crude gangliosides, it may be noted, are merely the water-soluble, hyperphasic fraction after a single partition dialysis of the chloroform-methanol extract. The typical range of sialic acid content is 17% to 22%; cod has only 11% sialic acid. The hexose to sialic acid ratios range from 2.4/2 to 4.4/2. The theoretical hexose to sialic acid ratio in the disialoganglioside, which has been found to be the major subfraction, is 3:2 (4). In the monosialo- and trisialogangliosides the ratios are 3:1 and 3:3 respectively. The high values of hexose to sialic acid ratios obtained may be due to the presence in the crude material of hexose-containing contaminating lipids such as sulfatides and cerebrosides.

Reproducibility of the Preparative Technique

Crude gangliosides were prepared from 3 individual ox brains and from 3 individual dog brains. The ox brains yielded 3.87, 3.75 and 3.88 mg of gangliosides per gram of wet gray matter with a sialic acid content of 17.7%, 16.1% and 19.3% respectively. The dog brains yielded 6.5, 5.4 and 4.8 mg of gangliosides per gram of wet gray matter with a sialic acid content of 14.6%, 16.6% and 17.5% respectively. The individual dog brains seemed more similar to each other when their content of sialic acid per dry weight of gray matter was calculated. On this basis (per cent dry weight) they contained 0.45%, 0.49% and 0.43% hyperphasic sialic acid.

Hyperphasic Preparations From Non-Cerebral Tissues

The procedure employed with cerebral tissue was used to prepare crude gangliosides from such tissues as cerebellum, spleen, lung, thymus, kidney, heart and the electric organ of the electric eel. In general, only in the cerebellum was a hyperphasic chromagen (giving a pink color with *p*-dimethylaminobenzaldehyde) observed in amounts comparable with cerebral gangliosides. It is of interest that the partition dialysis method confirms the presence of hyperphasic chromagen in spleen and kidney, first observed over 40 years ago (19,20).

The electric organ of the electric eel contributed an amount of material (to the water phase after partition dialysis) three or four times the amount usually found from brain. Sialic acid was found to be absent from this material by both the direct Ehrlich (8) and the 2-thiobarbituric acid (21) methods. This material contained 6.6% hexose and 1.9% phosphorus.

Cat, Dog, Pig and Sheep Ganglioside Subfraction Analysis

Ascending TLC of the total gangliosides of the gray matter of cat, dog, pig and sheep brains upon visualization with resorcinol spray showed 3 major subfractions for the gangliosides of the dog, pig and sheep, and 4 for the cat. The subfraction which showed the greatest mobility was a monosialoganglioside; the subfraction which migrated immediately behind was identified as a disialoganglioside. This subfraction appeared as the darkest spot and was therefore the one containing the most sialic acid. These two subfractions were the same for each species. For the dog, pig and sheep one subfraction was found to migrate more slowly than the disialoganglioside; for the dog and sheep it was found to be a trisialoganglioside while for the pig it was identified as a second disialoganglioside (using the criteria given below). The cat ganglioside gave 2 spots which migrated more slowly than the disialoganglioside; both of these were identified as trisialo compounds.

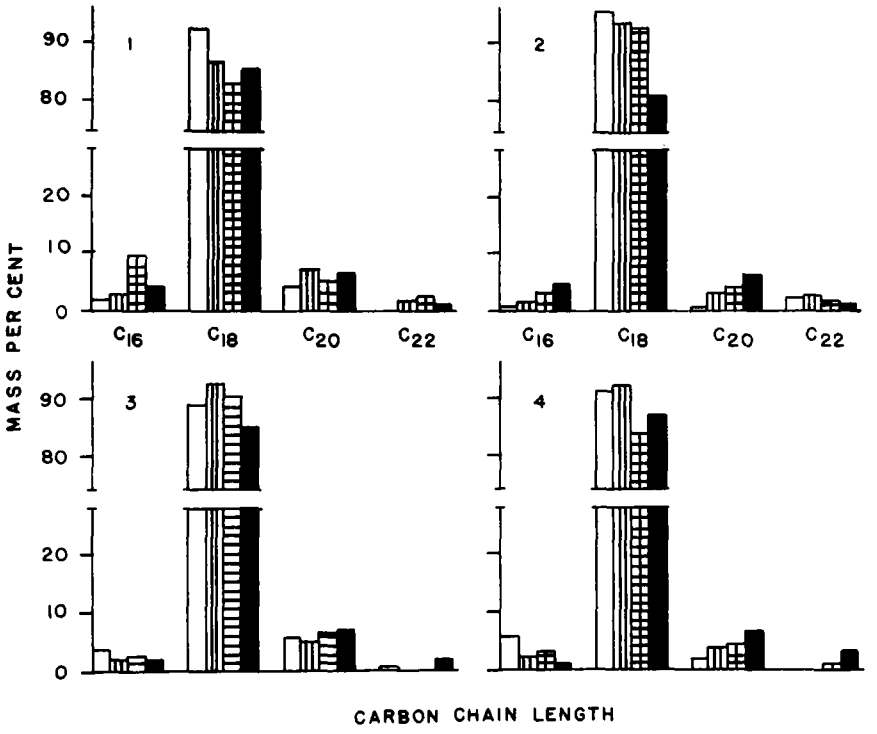

FIG. 2. Mass per cent of C_{16}, C_{18}, C_{20} and C_{22} found in 1, cat; 2, dog; 3, pig; and 4, sheep gangliosides. ☐ Monosialo-, ▥ disialo-, ▤ disialo-$_2$ (found in pig), ▦ trisialo- and ■ total ganglioside.

In addition to identifying the subfractions as monosialo-, disialo- or trisialogangliosides by their migration patterns on TLC, two other criteria were used. One was the sialic acid content of the isolated subfraction as determined on the unhydrolyzed sample, and the second was the sialic acid to hexose ratio. The sialic acid to hexose ratios obtained are shown in Table II.

Qualitative identification of the sugars indicated only glucose and galactose. Qualitative identification of the hexosamine moiety indicated only galactosamine. The ratios of N-acetylgalactosamine-galactose-glucose were found to approximate 1:2:1 for each subfraction. In Table III can be seen the percentages and the ratios obtained for the cat brain subfractions and for the total gangliosides obtained from each species. It can be seen that the crude cat gangliosides show a ratio of 1:3:1. The reason for the variation of this ratio from the expected ratio of 1:2:1 may be the presence of contaminating hexose-rich lipids, such as sulfatides and cerebrosides. This may also be the reason for the higher than expected ratios of hexose to sialic acid obtained for some of the crude preparations of gangliosides (Table II). The hexose to sialic acid ratio obtained for the total gangliosides of dog brain was found to decrease (toward the theoretical value) upon further purification of the total gangliosides.

The major fatty acids found in the various gangliosides subfractions are shown in Figure 2. It was found that the major component (80% or more) was C_{18}, stearic acid, in each sample. Arachidic acid, C_{20}, was found to be present in the next greatest quantity with lesser amounts of palmitic, C_{16}, and behenic acid, C_{22}, also found. It was found that the total gangliosides contained small amounts (less than 2%) of C_{24} and C_{26} fatty acids.

The results of the oxidation and subsequent formation of the methyl esters of the fatty acids obtained from the sphingosine samples prepared are reported in Table IV. It can be seen that the C_{14} to C_{16} ratio decreases as one goes from a monosialo-, to a disialo-, to a trisialoganglioside. This would indicate that the amount of icosisphingosine increases and predominates in the trisialo compound, while sphingosine is the major component of the monosialoganglioside.

Only single samples of the ganglioside subfractions were available for sphingosine

TABLE IV

Ratio of the C_{14} Methyl Ester to the C_{16} Methyl Ester Obtained From Oxidation of Sphingosine From Cat, Dog, Pig and Sheep Brain Gangliosides

Ganglioside fraction	Ratio C_{14}:C_{16}			
	Cat	Dog	Sheep	Pig
Monosialo-	80:20	74:26	71:29	74:26
Disialo-	50:50	53:47	37:63	48:52
Disialo-$_2$	----	----	----	29:71
Trisialo-	1:99	34:66	39:61	----
Trisialo-$_2$	22:78	----	----	----
Total (9)	53:47	48:52	43:57	51:49
Total (16)	----	48:52	49:51	44:56

analysis. Duplicate sphingosine analyses, however, were performed on the total gangliosides. Sphingosine was determined both by the method of Ledeen and Salsman (9) and by that of Rosenberg and Stern (16). Both preparations were oxidized in the same manner. It can be seen in Table IV that the results obtained agree with each other and that there are approximately equal amounts of sphingosine and icosisphingosine in the total gangliosides of the 4 species.

Thin Layer Chromatography of Cod Ganglioside

All ganglioside preparations were extracted from wet tissue by chloroform-methanol; they then passed into the aqueous phase, from which they were not dialyzable. When purification of cod brain gangliosides was attempted by repeating the isolation procedure, it was found that the material was no longer soluble in chloroform-methanol. The gangliosides of eel brain behaved similarly.

Thin layer chromatography of the total gangliosides of cod brain showed that the major sialic acid-containing fractions migrated more slowly than the gangliosides (even the trisialogangliosides) of the other species studied. In fact, a major portion (about one fourth) of the sialic acid was recovered from the origin portion of the thin layer chromatogram. This migration behavior might indicate the presence of a polysialoganglioside or some other sialic acid rich compound or aggregate of high polarity.

DISCUSSION

Of the lipids already characterized, gangliosides are among the most complex. A trisialoganglioside, for example, may contain 7 polar and 2 nonpolar units; many variants are possible in the position and structure of these 9 units. The structural complexity of the gangliosides and their ambivalent solubility (they are soluble in both aqueous and organic solvents) may be related to the function of gangliosides in neural tissue (22,23). From the present study it appears that no brain studied lacks gangliosides, that the total population of ganglioside molecules is grossly constant in the species studied, and that the details of ganglioside composition and structure differ from species to species.

Lower species might be expected to reflect in some way a more primitive stage of ganglioside evolution (24). In this study, using sialic acid as an index, no obvious phylogenetic trend was observed. The study was limited, of course, to one subphylum, since only animals with brains large enough for handling on the gram scale were investigated. The electric eel, frog and dog each had about 20% sialic acid in crude ganglioside preparations. Crude gangliosides from cod brain did have some resemblance to the early stages of chick embryo brain (2) with respect to behavior upon redistribution and somewhat lower content of sialic acid.

Variation in ganglioside polarity is mediated by such differences as the number of sialic acid residues and the length of hydrocarbon chains in the fatty acid and the sphingosine moieties. Kuhn et al. (25) first isolated and identified the 4 major ganglioside subfractions of a normal brain. They found a monosialoganglioside, 2 isomeric disialogangliosides and a trisialoganglioside. The molar ratios of glucose-galactose-N-acetylgalactosamine were 1:2:1 for all 4 fractions. In the present work, the major ganglioside subfractions were also monosialo-, disialo- or trisialoganglioside compounds. The ratio of glucose-galactose-N-acetylgalactosamine was also about 1:2:1 in each instance. The sialic acid-hexose ratios indicate that in the gray matter of a cat brain there are two major trisialo- fractions, each having the same composition. The existence of several trisialogangliosides has been noted by others (26,27). The

apparent isolation of only one disialoganglioside in most cases does not preclude the presence of 2 isomeric substances. It may be that under the conditions used to isolate the subfractions the two disialoganglioside compounds were not separated, or that the second one was present in a minor amount.

Large amounts of stearic acid were found in all the ganglioside subfractions studied. Kishimoto and Radin (28) have found stearic acid to account for over 80% of the fatty acids present in gangliosides and have proposed that this be used as a criterion of ganglioside purity. It may be that the enzymes responsible for the incorporation of fatty acids into the gangliosides are specific for stearic acid. The present study shows that the minor fatty acid components of the gangliosides do not vary in a way suggestive of a trend. Minor fatty acids may find their way from a (diet dependent) metabolic pool into the gangliosides due to an incomplete specificity of the enzymes responsible for ganglioside biosynthesis. The presence of C_{24} and C_{26} fatty acids in the crude ganglioside preparations may be due to contaminating lipids, such as cerebrosides and phospholipids, both of which were identified by TLC in total ganglioside preparations. Together with the C_{18}, the minor components C_{16}, C_{20} and C_{22} were identified in most subfractions, and would appear to be true components of the gangliosides.

The observed pattern of decreased sphingosine and increased icosisphingosine in the gangliosides of higher sialic acid content may indicate that gangliosides rich in icosisphingosine and those rich in sialic acid are related either structurally or by a common biosynthetic pathway.

ACKNOWLEDGMENTS

Supported by a Research Grant from the National Institutes of Health, U.S. Public Health Service, No. NB-03085-06. C. -L. Schengrund held a Seton Hall University Pre-doctoral Research Fellowship.

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[Received August 29, 1968]

The Distribution and Excretion of Tritium Labeled Cerebroside (Galactosyl Ceramide) Radioactivity by the Monkey[a]

ROBERT MAIN BURTON, Department of Pharmacology and Beaumont-May Institute of Neurology, Washington University School of Medicine, St. Louis, Missouri 63110, **WALTER LEWIS III** and **MARY ANN SODD**, Section on Lipid Chemistry, National Institutes of Neurological Diseases and Blindness, National Institutes of Health, Bethesda, Maryland 20014

ABSTRACT

Tritium labeled galactosyl ceramide was administered intravenously to three male monkeys (*Macaca mulatta*). Blood, urine, feces and organs obtained at autopsy were examined for radioactivity after 1 hr, 8 hr and 37 days. The radioactive galactosyl ceramide was taken up by the reticuloendothelial system. Very little radioactivity appeared in brain tissue. The radioactivity was excreted primarily in urine.

INTRODUCTION

The glycolipid cerebroside occurs widely in animal tissue but appears in white matter of the central nervous system in the largest amounts as galactosyl ceramide, i.e., about 5% of the wet weight. Abbreviations and nomenclature used in this paper are presented in "Lipid and Lipidoses" (1).

Gaucher's disease is characterized by the accumulation of large amounts of cerebrosides (as glucosyl ceramide) in cells of the reticuloendothelial system. In this disease the central nervous system appears not to be affected; no abnormal amounts of cerebrosides have been seen in brain tissue. Lesions similar to those seen in Gaucher's disease (histological and chemical) were produced in the rabbit by administering large amounts of galactosyl ceramide (2). Similar experiments in which large amounts of galactosyl ceramide were given daily intraperitoneally to rats over a long period of time failed to show tissue accumulation of cerebrosides (Burton, unpublished observations). Since the amount of cerebroside required to produce lesions in rabbits was large (0.5 g daily intraperitoneal or 1 g daily orally) and the dose which failed to produce tissue accumulation in the rats was large (0.2-4.8 g daily intraperitoneally), it is indicative of a rapid elimination of cerebrosides by metabolism or excretion. To study this aspect in greater detail, radioactive galactosyl ceramide was prepared and administered in small amounts to monkeys. Blood, urine and feces collected daily and tissues at autopsy were examined for radioactivity and cerebrosides. The details of these experiments are reported in this paper.

MATERIALS AND METHODS

Cerebrosides (galactosyl ceramides) were isolated from beef brain by the procedure of Radin et al. (3).

Cerebroside (500 mg) was exposed to 2 c tritium gas at 0.38 atm and room temperature for two weeks [Wilzbach, (4); service performed by New England Nuclear Corp., Boston]. Exchangeable tritium was removed by repeated crystallization from methanol. The labeled cerebroside was passed over Dowex-1, Dowex-50 and Florisil (3). It was chromatographed on silicic acid and eluted with chloroform-methanol (10:1 v/v) [see Figure 3 (5)]. The specific activity of ^{3}H-cerebroside is 1.2 x 10^6 cpm/mg; the yield, 89%. Both cerebroside and ^{3}H-cerebroside are white, waxy solid with the same melting point both separate and mixed (mp 178 C uncorrected, Fisher-Johns unit). X-ray diffraction powder patterns for both the normal and tritium label cerebrosides are identical (Fig. 1). While cerebroside hydrates readily on heating with water and forms a smooth, stable colloid, the ^{3}H-cerebroside hydrates with difficulty and does not form a stable colloid. ^{3}H-Cerebroside was hydrolyzed (3.5 N HCl 18 hr reflux) and the products subsequently separated by organic solvent-aqueous phase partitioning with acid and alkaline aqueous phases. The sphingosine, fatty acids and ceramides were identified by silicic acid chromatography. The ceramides were recycled through the procedure. The sphingosine was quantitated by the procedure of Brady and Burton (6) and the fatty acids by titration. The galactose was identified by paper chromatography (7) and quantitated by the anthrone procedure of Radin et al. (3). The products, galactose, sphingosine, ceramide and fatty acids showed a random labeling pattern approaching the theoretical distribution (41.0% sphingosine, 54.6% fatty acids and 4.0% galactose found;

[a]Presented at the 137th Meeting of the American Chemical Society.

theory 39.3%, 54.0%, as lignoceric acid, and 6.7%).

^{3}H-Cerebroside (40 mg) was suspended in 25 ml normal saline at 100 C and cooled. This was injected into a left leg vein of the pentobarbital anesthetized male monkey (*Macaca mulata*; 2.5 kg body weight) and followed immediately by a slow intravenous infusion of 1000 ml normal saline to promote diuresis. An inlieing catheter was used for total urine collections. Monkey D was given penicillin and chloromycetin prophylactically because of the danger of infection due to the catheter being in place for an extended period of time. Blood samples (2 ml) were drawn from the right leg vein at frequent intervals, i.e., every minute for the first 5 min, every 5 min for 1 hr, then every 30 min for 8 hr, and three times weekly for 37 days. Organ weights and tissue samples were obtained at autopsy of each monkey. Monkey B was killed by exsanguination 1 hr after ^{3}H-cerebroside administration, monkey C, 8 hr, and monkey D, 37 days later. Tissue samples were processed as described earlier (5). Cerebrosides were isolated by the method of Radin et al. (3). Radioactivity was determined with the Packard Tri-Carb Liquid Scintillation Spectrometer, when necessary correcting for quenching by the use of internal standards. Tissue was dissolved in Hyamine base [Hyamine 10 - x base, p-(diisobutylcresoxy ethoxy ethyl) dimethylbenzylamine] prior to counting, and urine was counted in toluene-ethanol mixture (6).

RESULTS

Blood Plasma Level and Urinary Excretion of Radioactivity From ^{3}H-Cerebroside.

Immediately following the injection of ^{3}H-cerebroside, the high blood radioactivity began to fall (Fig. 2). Within one to two days, two thirds of the radioactive cerebroside had been removed from the blood. After the third day, the level of radioactivity in the blood plasma began to rise to a new level at 8 days, remaining constant until the 25th day post injection. Experiments have shown that the radioactivity in blood resides with the plasma fraction, little or no radioactivity being associated with the red blood cells. The urinary excretion of radioactivity was rapid during the period of saline infusion, but after the first day, dropped to a low, constantly decreasing, rate of excretion. During the 37 days, monkey D excreted 60% of the total injected radioactivity in urine. In contrast to blood plasma where most of the radioactivity appears to be protein bound ^{3}H-cerebroside, the tritium in urine is present to a large extent as water and water-soluble unidentified compounds. No radioactivity was found in the feces.

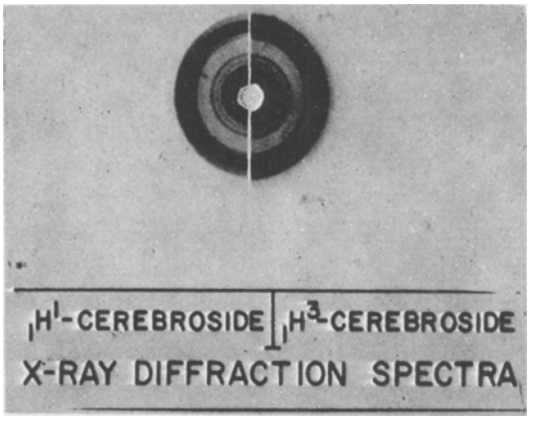

FIG. 1. Comparison of galactosyl ceramide with tritium-labeled galactosyl ceramide.

Tissue distribution of radioactivity derived from ^{3}H-cerebroside is shown by the data in Table I. The data presented in Table I indicate the range of organ weights for the three monkeys and the total radioactivity for many of the organs. The recovery of radioactivity for the monkeys ranged from 96% to 107%. The tissues that contained the bulk of the radioactivity in order of decreasing amounts, were lung, liver, spleen, blood plasma and kidney. However, on a specific activity basis the order of activity decreased as follows: lung, spleen liver and kidney (Monkey B, 5000,60,71,31: monkey D, 1100,144,54,18 cpm/mg wet weight). Other tissues such as adrenals, pancreas, heart, skeletal muscle, testes, lymph nodes and blood vessels contained little radioactivity.

During the 37 day interval following the injection, radioactivity in the spleen increased over two fold even though 60% of the total tritium administered had been excreted in the urine. The level of tritium in the liver decreased on a specific activity basis, but increased 85% on a total organ basis. Abdominal fat, which had no radioactivity at the 1 hr interval contained tritium after 37 days. Bone marrow also showed a marked increase in radioactivity. The results indicate that little radioactive cerebroside is taken up by the central nervous system tissue (Monkey D, 0.1-1.1 cpm/mg wet weight).

Of particular interest is the large amount of radioactivity in lung tissue which decreases with time. In view of the difficulty with which ^{3}H-cerebroside hydrates, it seems probable that lung tissue mechanically retained larger particles of ^{3}H-cerebroside. Additional evidence

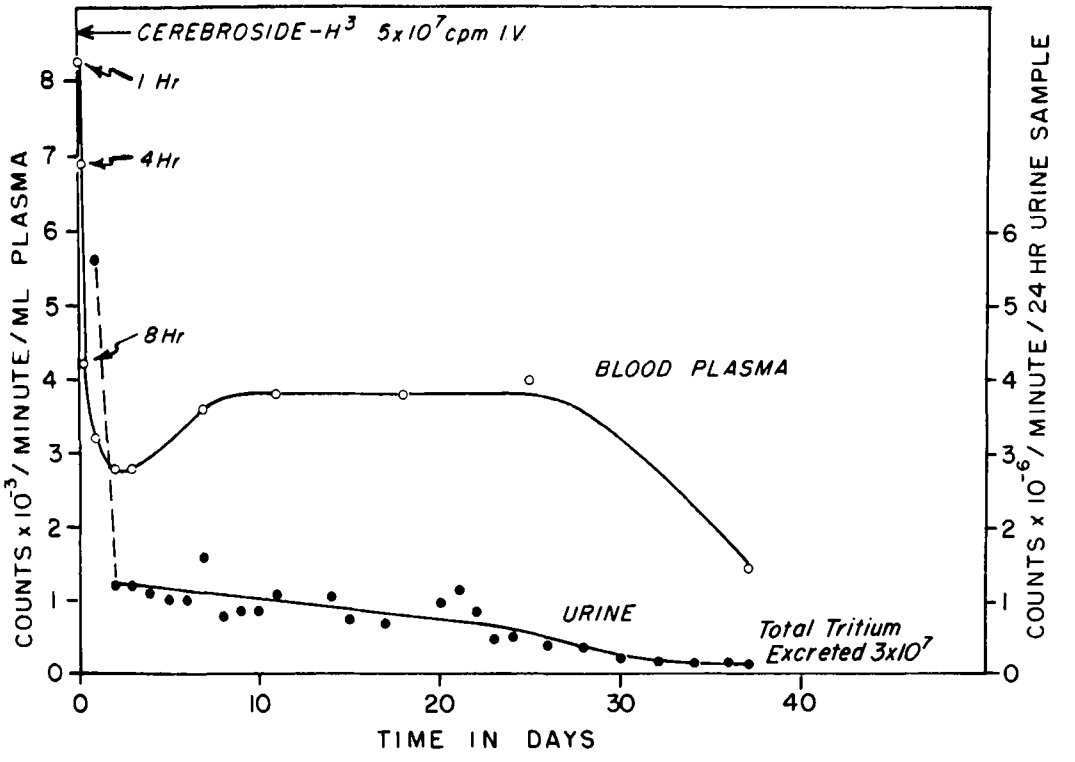

FIG. 2. Blood and urine radioactivity from administered ^{3}H-cerebroside. This linear plot presents the data in the most convenient form to emphasize the minimum value at day 1 and 2 and the constant blood radioactivity from day 8 to day 25. A plot of the log radioactivity vs. tissue, yields $t_{1/2}$ (initial blood level) = 7.5 hr and a $t_{1/2}$ (urine) = 32.5 days.

which indicated that this explanation is probably valid, was obtained from similar experiments using rats. The ^{3}H-cerebroside was suspended in hot water and cooled. It was administered by subcutaneous injection in the upper dorsal region. The radioactivity in these experiments was distributed in a similar manner to the monkey, except that the concentration in lung tissue was less than that in the liver and spleen. In the monkey experiments reported in Table I, the lung tissue ^{3}H-cerebroside was 2-to 20-fold higher than the liver radioactivity on a total activity basis and was even more dra matically higher on a specific activity basis.

When the tissues from monkeys B and C (killed 1 hr and 8 hr post injection) were examined, all of the radioactivity was present as cerebroside. However after 37 days (monkey D) the radioactivity present as cerebroside was considerably reduced. Some of the tritium appeared as water soluble components or as water itself. The radioactivity in adipose tissue was primarily glycerides. Other tissues contained some glycerides and phospholipids, however a large part of the radioactivity was not removed with the neutral lipids and phospholipids. This residual radioactivity was shown to consist entirely of cerebrosides.

DISCUSSION

The introduction of recoil labeling of compounds by tritium has made available many radioactive compounds whose synthesis are difficult (4). The use of these randomly labeled compounds in biology must be approached with caution; there is danger of decomposition products being present and of abnormal effects due to the substitution of tritium for hydrogen with the consequent alteration in mass and molecular radii. In these studies, the tritium-labeled cerebroside appeared normal in all aspects examined (isolation procedures, chromatographic behavior, x-ray powder diagram and melting point) except that the tritium labeled cerebroside had a more crystalline appearance and failed to hydrate as readily as unlabeled cerebroside. These experiments are a study only of the fate of tritium labeled galactosyl ceramide in the monkey.

TABLE I

Monkey Tissue Weights and Total Radioactivity

Tissue	Wet weight (Range)	Total radioactivity Monkey B (1 hr)	Monkey C (8 hr)	Monkey D (37 days)
Monkey[a]	g 2500-2530	cpm[b] 55 x 10^6	cpm[b] 40 x 10^6	cpm 55 x 10^6
Spleen	4-4.9	335 x 10^3	406 x 10^3	703 x 10^3
Liver	51-110	3,670 x	4,000 x	6,740 x
Kidneys	8-12	372 x	102 x	153 x
Adrenals	1		6 x	153 x
Pancreas	1	---	---	---
Testes	1	---	1 x	---
Lymph nodes	1	---	4 x	---
Adipose	296-490[c]	0	76 x	338 x
Bone marrow	5[c]	5 x	11 x	178 x
Lung	11-15	53,336 x	27,200 x	15,150 x
Skeletal muscle	1100[c]	---	836 x	---
Heart muscle	7-9	108 x	13 x	18 x
Cerebrum, total	71-74	144 x	44 x	17 x
Cerebellum total	8-9	7 x	3 x	0.4 x
Spinal cord	5	1 x	0.7 x	2 x
Blood plasma	100-109	964 x	508 x	135 x
Urine	---	---	5,068 x	30,000 x
Total	1671-1991	58,942	38,280	53,434
Per cent of administered cerebroside	---	107	96	97
	67-79[d]	---	---	---

[a]Body weight and cerebroside radioactivity administered.

[b]All of the radioactivity was present as ^{3}H-cerebroside, except for 13% excreted in urine as water and water soluble compounds.

[c]Approximation of total body tissues.

[d]Per cent of monkey tissues recovered. The skin, skeleton and other tissues not tabulated were disposed of by incineration.

These experiments show that ^{3}H-cerebroside is associated with a protein fraction of blood plasma rather than with the blood cells. ^{3}H-Cerebroside is removed from the blood and deposited in tissues, especially of the reticuloendothelial system. Autopsy of two monkeys (1 hr and 8 hr post injection) show that the tissue radioactivity is present as galactosyl ^{3}H-ceramide. Little access to the central nervous system is gained by periferal ^{3}H-cerebroside. This is consistent with the pathology observed in Gaucher's disease; however the cerebroside accumulated in this disease is glucosyl ceramide, not galactosyl ceramide. Kopaczyk and Radin (8) have shown that emulsified cerebrosides injected into the brains of rats can be metabolized by this tissue.

^{3}H-Cerebroside is metabolized by the monkey as indicated by tritium containing water and water soluble compounds in the urine and the appearance of non-cerebroside radioactive compounds in the organs examined. Radioactivity found in the brain after 37 days occurs as lipids other than cerebrosides. The initial rapid loss of blood radioactivity must reflect a distribution of cerebrosides to the tissues. The half life for this initial phase is $t_{1/2}$ = 7.5 hr. The reason for the minimum occurring in the radioactivity of blood plasma at 3 and 4 days is unknown (Fig. 2). It could represent a redistribution of the ^{3}H-cerebroside or a period of induction or unmasking of ^{3}H-cerebroside metabolizing enzymes. The constant blood level of radioactivity between the eighth and 26th day reflects the release of ^{3}H-cerebroside and metabolites from tissue depots. The urinary pattern of tritium excretion reflects a complex metabolic picture; the net result of the total degradation of ^{3}H-cerebroside to tritium containing water and water soluble compounds. The rate of urinary excretion of tritium is constantly decreasing, with a half life of 32 days, which indicates that the metabolic system for the total degradation and excretion of ^{3}H-cerebroside has not been saturated by the amount of glycolipid administered.

Thus, these in vivo experiments are consistent with the studies of Brady et al. (9,10), Gatt (11), and Hajra et al. (12) which show the presence of hydrolytic enzymes capable of hydrolyzing cerebrosides.

ACKNOWLEDGMENT

Supported in part by U. S. Public Health Service Grant No. NB-01575-11 from the National Institute of Neurological Diseases and Blindness.

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[Received December 16, 1968]

Essential Fatty Acid-Deficient Rats: I. Growth and Testes Development

E. AAES-JØRGENSEN and **G. HØLMER**, Department of Biochemistry and Nutrition, and the Danish Fat Research Institute, Polytechnic Institute, Lyngby, Denmark

ABSTRACT

Partially hydrogenated oils as the sole dietary fat enhances the development of essential fatty acid (EFA) deficiency in young rats. Partially hydrogenated herring oil (HHO) caused total impairment of the spermatogenic tissue after five weeks of experiment, while partially hydrogenated arachis oil (HAO) caused severe degeneration of this tissue in 15 weeks. A fat-free diet caused degeneration in 26 weeks. In the dietary fats, the total content of *trans* acids, calculated as elaidic acid, was 47% and 23% in HAO and HHO, respectively. Further, varying amounts of different positional isomeric fatty acids were also present in the partially hydrogenated oils. Besides the specific tissue changes, poor growth, poor feed efficiency and skin signs characteristic of EFA deficiency were noticed. On the other hand, partially hydrogenated soybean oil (HSO) as the sole dietary fat kept the animals normal in all respects. This oil still contained 32% linoleic acid; the total content of *trans* acids amounted to 11%, calculated as elaidic acid.

INTRODUCTION

Essential fatty acid (EFA) deficiency in weanling male rats causes very severe impairment of the spermatogenic tissue after about 24 weeks on a fat-free diet (1-3). It is our experience that diets containing partially hydrogenated vegetable oils as the sole dietary fat enhance the degeneration of the spermatogenic tissue as compared to fat-free diets (3-5). It should be mentioned that all the effects of feeding partially hydrogenated oils are prevented by the presence of small amounts of essential fatty acids. In the present studies, we are reporting on experiments comparing the effects on growth and testes development of weanling male rats fed on partially hydrogenated vegetable oils or a partially hydrogenated herring oil. Thus, the fatty acid spectrum in the experimental dietary fats is quite different. This fact may be of interest in case the fatty acid pattern as such, or the isomeric unsaturated fatty acids, are of importance for the degenerative changes in the spermatogenic tissue in EFA deficiency. Details on the fatty acid composition of the dietary fats and of the different lipid classes of the testes will be given in forthcoming publications (6,7).

EXPERIMENTAL PROCEDURES

One hundred and fifty weanling male rats were divided into five groups of 30 animals. Each of these groups was further divided into three subgroups of 10 animals. One subgroup from each of the main experimental groups was killed after 5, 15 and 26 weeks, respectively. Group 1 received a fat-free diet of the following composition: Vitamin Test Casein (Genatosan Ltd., Loughborough, England), 20%; sucrose, 74%; vitamin mixture [0.5 g of the vitamin mixture consisted of: biotin, 0.05 mg; folic acid, 0.2 mg; para-aminobenzoic acid, 1 mg; riboflavin, 1.5 mg; pyridoxine, HCl, 1.5 mg; thiamin, HCl, 1.5 mg; inositol, 5 mg; niacinamide, 5 mg; calcium pantothenate, 5 mg; ascorbic acid, 10 mg; tocopherol acetate, 5 mg (as Ephynal acetate, F. Hoffmann-La Roche & Co.); vitamin K, 0.5 mg (as Synkavit, F. Hoffmann-La Roche & Co.); vitamin B_{12}, 0.003 mg (as Bendogen, GEA); vitamin A, 1000 IU, and vitamin D_3, 100 IU (as Rovimix AD_3, 50/5, F. Hoffmann-La Roche & Co.); and sucrose, 500 mg], 0.5%; salt mixture [100 g of the salt mixture contained: NaCl, 6 g; Mg $(HCO_3)_2$, 5 g; HCl, 7 g; K_2HPO_4, 17 g; NaH_2PO_4, 2 H_2O, 10 g; Ca $(H_2PO_4)_2$, 2 H_2O, 17 g; Ca-lactate, 5 H_2O, 36 g; ferric citrate, 2 g. The following trace elements were added per 100 g of salt mixture: $Ca(IO_3)_2$, 6 H_2O, 5.81 mg; zinc carbonate, basic (56% Zn), 72 mg; $MnCO_3$ (47.8% Mn), 42 mg, NaF, 40 mg; $NaMoO_4$, 2 H_2O, 5 mg; $Cr_2(SO_4)_3$, 15 H_2O, 1.3 mg; SeO_2, 0.32 mg. In total: 185.43 mg per 100 g of salt mixture.] 5.0%; and choline chloride, 0.5%. Groups 2, 3, 4 and 5 received the fat-free diet in which 28 wt % of sucrose was replaced by 28 wt % of partially hydrogenated arachis oil (HAO) (Aarhus Oliefabrik Inc., Aarhus, Denmark), partially hydrogenated soybean oil (HSO) (Aarhus Oliefabrik Inc.), normally refined arachis oil (AO) (Aarhus Oliefabrik Inc.) or partially hydrogenated herring oil (HHO) (Dansk Sojakagefabrik Inc., Copenhagen, Denmark), respectively.

Diets and water were provided ad libitum. The animals were weighed and inspected week-

TABLE I

Fatty Acids of Dietary Oils (Wt %)

Fatty acids, per cent of oil	Partially hydrogenated arachis oil (HAO)	Partially hydrogenated soybean oil (HSO)	Arachis oil (AO)	Partially hydrogenated herring oil (HHO)
Saturated	29.3	19.8	19.1	41.9
Monoenoic	67.0[a]	41.4[b]	50.9[c]	44.8[d]
Polyenoic	3.3	39.0	29.9	13.2
$C_{18:2}$, ω6 Linoleic acid	0.8	31.8	28.8	0.6
$C_{18:3}$, ω3 Linolenic acid	—	3.4	1.1	—
Total trans acids (as Elaidic acid)	47	10.7	0	22.9

[a] $C_{16:1}$, 1%; $C_{18:1}$, 64.8%; $C_{20:1}$, 1.2%.
[b] $C_{16:1}$, 0.6%; $C_{18:1}$, 40.8%.
[c] $C_{16:1}$, 0.3%; $C_{18:1}$, 48.5%; $C_{20:1}$, 1.7%.
[d] $C_{16:1}$, 5.6%; $C_{18:1}$, 13.0%; $C_{20:1}$, 11.3%; $C_{22:1}$, 14.1%.

ly. On autopsy, after 5, 15 and 26 weeks, respectively, the left testis was removed, weighed and frozen for lipid analysis (6,7), while the right testis and epididymis were fixed in 4% formaldehyde for histological examinations.

RESULTS AND DISCUSSION

Dietary Fats

The dietary fats were all commercial products. However, the partially hydrogenated soybean oil was hydrogenated by means of a special technique, which primarily eliminated the high content of trienoic acid from the original oil. Gas chromatographic analyses of all the dietary fats are published in detail in a following publication (6).

A summary of the composition of the dietary fats is shown in Table I. The greatest discrepancies in composition are between the HHO and the other three dietary fats. HHO has a large content of different monoenoic fatty acids, especially $C_{20:1}$ and $C_{22:1}$ (6). In the HAO, the unsaturated fatty acid picture is dominated by $C_{18:1}$. The HSO and the AO contain about equal amounts of mono- and dienoic acids.

In the partially hydrogenated oils the contents of *trans* acids are considerable. The amount of these fatty acids in HSO is half that of HHO and one quarter that of HAO. The *trans* acid in HAO and HSO is practically all elaidic acid. In HHO, the *trans* acids comprise about equal amounts of $C_{18:1}$, $C_{20:1}$ and $C_{22:1}$. Further, several other isomers are also present, e.g., *cis*, *trans* $C_{20:2}$ and $C_{22:2}$, and *cis,cis,trans* 20:3 and 22:3. The content of conjugated fatty acids appears negligible in all four experimental fats.

Growth Rates and Skin Signs

On the EFA-deficient diets, growth rates began to level off after five to seven weeks, i.e., Groups 1, 2, and 5 (Fig. 1). The animals on the fat-free diet resumed growth during the 11th through the 14th week, and then again leveled off at a somewhat higher plateau. In contrast, the animals fed HAO or HHO gained very little weight from the 8th through the 25th week. The former of the two fats had twice as much *trans* acids as the latter (Table I), and both were very low in linoleic acid. The HSO contained even more essential fatty acid than the AO (Table I). The animals fed on the HSO diet grew slightly more than the controls fed AO. These results indicate that a possible stressing effect of partially hydrogenated dietary oils was eliminated or counteracted by the presence of EFA.

The scaliness of the feet, tail and skin did not develop to any great extent (Table II). The humidity in the animal quarters varied from 30-40% during the last two months. The yellow-brown pigment on the back of the animals developed normally in the non-EFA-deficient animals, Groups 3 and 4, but did not, or practically not, occur in the EFA-deficient rats (Table II).

Food Consumption

Measurement of food intake in the various groups was carried out through the 9th and 10th week, and again through the 24th and 25th week (Table II). The highest caloric con-

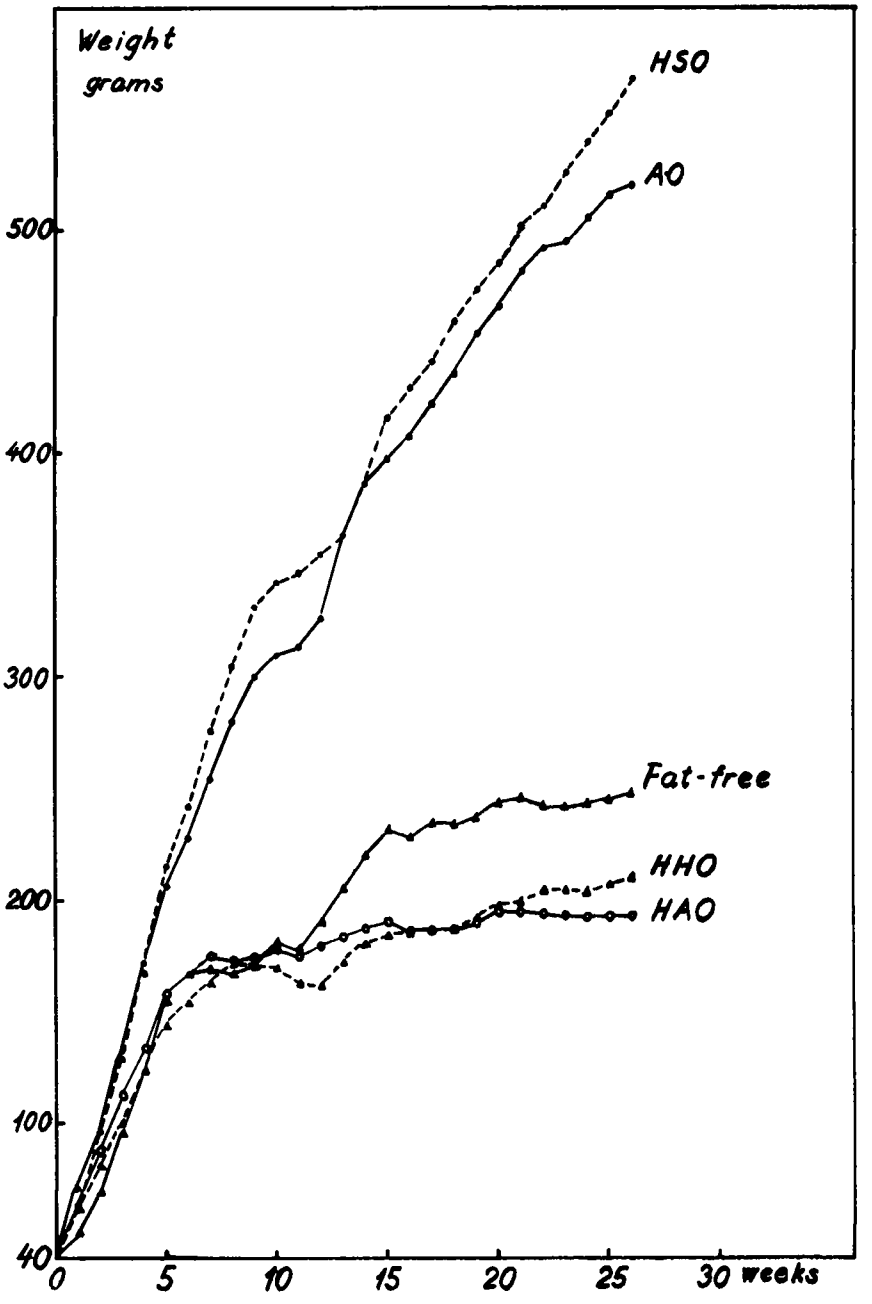

FIG. 1. Average growth rate of male rats fed on a diet with 28% partially hydrogenated soybean oil (HSO), 28% partially hydrogenated herring oil (HHO), 28% partially hydrogenated arachis oil (HAO), 28% arachis oil (AO), or a fat-free diet (Fat-free).

sumption during both periods was observed in the two groups fed AO or HSO (Table II). These animals also grew the most (Fig. 1). The weight increase in these animals was to a great extent due to very large quantities of depot fat.

The caloric intake per square meter surface during both testing periods (Table II) was greatest for the rats fed HAO or HHO, respectively. At the end of the experiment, a somewhat similar picture was obtained with the animals on

TABLE II

Scaliness, Pigmentation and Food Consumption Measurement

Group no.	Diet characteristics	Scaliness[a]		Pigment[b]		Food intake/animal/day					
						Average 8th-9th week			Average 24th-25th week		
		9th week	26th week	9th week	26th week	grams	calories[c]	calories/ sq.m/surface[d]	grams	calories[c]	Calories sq.m/surface[d]
1	Fat-free	1.8	1.2	0	0	11.0	41.4	1186	13.1	49.3	1105
2	Partially hydrogenated arachis oil (HAO)	1.7	1.5	0	0.7	10.0	51.6	1453	9.3	48.0	1270
3	Partially hydrogenated soybean oil (HSO)	0	0	1.7	2.4	12.6	65.0	1195	14.2	73.3	959
4	Arachis oil (AO)	0	0	1.7	2.1	11.2	57.8	1135	11.2	60.9	841
5	Partially hydrogenated herring oil (HHO)	1.5	1.1	0	0.6	9.7	50.1	1446	9.9	51.1	1281

[a]Dermal symptoms were scored with a range of 0 to 3 each for tail, forelegs, hindlegs and dandruff, and the average of these four scores per animal was calculated for the group.

[b]Yellow-brown pigment on the back of the animals was scored 0 to 3.

[c]Calculated by assuming that carbohydrate and protein yield 4 Cal/g, and fat 9 Cal/g.

[d]The surface area is calculated by the formula: surface area (sq.m) = $11.63\sqrt[3]{w^2}$, where w is weight in grams (17,18).

the fat-free diet (Table II). These data compared to those obtained with animals fed AO or HSO illustrate the poor caloric efficiency in the EFA-deficient animals. This corroborates the findings of previous experiments from this laboratory (8,9). The reduced caloric efficiency may possibly be explained, at least to some extent, as an uncoupling of the oxidative phosphorylation process (10-15). It is tempting to speculate that the present results may partially be explained as an effect of the fatty acid pattern of the dietary partially hydrogenated oils. Thus, the affinity for isomeric unsaturated fatty acids, e.g., in the build up of lipoproteins, may be much less than for EFA, and for the isomeric acids synthesized in EFA-deficiency. The incorporation of isomeric unsaturated fatty acids into lipoproteins may result in drastic changes in the physico-chemical characteristics of membranes and enzyme moieties, thereby directly influencing formation of normal cells, e.g., in spermatogenesis, or indirectly by disturbing the normal course of oxidative phosphorylation. However, the rather similar data obtained after long-term experiments with animals fed on the fat-free diet indicate that, apart from a possible specific role of dietary isomeric unsaturated fatty acids, a similar effect eventually results from the disappearance of EFA, which favors the biosynthesis of large quantities of positional isomers of unsaturated fatty acids.

Testes: Weight and Histology

On gross examination, no differences in size were observed between the left and the right testes from the same animal in any of the experimental groups.

After five weeks of experiments, the absolute and relative weights of the left testis from the animals fed HHO were significantly lower than those of any of the other groups (Table III). Histological examination of the right testis from the former animals revealed an almost complete degeneration of the spermatogenic tissue (Table III).

The fat-free diet and that with HAO also caused a remarkably lower absolute weight of the testis after five weeks compared to that of the EFA supplemented animals (Table III).

During the following 10 weeks the absolute and the relative weights of the testis decreased in the animals fed no fat or 28% HAO (Table III). The decrease in relative weight is to some extent explained by a weight increase in the animals on the fat-free diet (Group 1, Fig. 1), whereas the animals fed 28% HAO hardly gained in weight during this period (5th-15th week, Fig. 1). The decrease in testis weight

TABLE III

Testes: Average Weight of the Left Testis and Histological Examinations of the Right Testis and Epididymis of the Rats

Group no.	Diet characteristics	Left testis: weight (g)						Average degree of degeneration of spermatogenic tissue of *right testis and epididymis*[a]		
		5 weeks		15 weeks		26 weeks				
		absolute	relat.[b]	absolute	relat.[b]	absolute	relat.[b]	5 weeks	15 weeks	26 weeks
1	Fat-free	1.08 ± 0.04	0.70	0.94 ± 0.07	0.42	0.69 ± 0.04	0.28	1.0	2.5	3.9
2	Partially hydrogenated arachis oil	0.93 ± 0.04	0.61	0.70 ± 0.07	0.39	0.64 ± 0.05	0.33	1.6	4.0	4.9
3	Partially hydrogenated soybean oil	1.25 ± 0.03	0.63	1.51 ± 0.06	0.39	1.54 ± 0.04	0.28	0	0	0
4	Arachis oil	1.22 ± 0.06	0.62	1.55 ± 0.05	0.43	1.55 ± 0.07	0.30	0	0	0
5	Partially hydrogenated herring oil	0.49 ± 0.04	0.34	0.59 ± 0.03	0.35	0.76 ± 0.04	0.41	4.7	5	5

[a]Assessed from a scale graduated from 0 (no degeneration) to 5 (total degeneration) (3).
[b]Percentage of body weight.

occurred simultaneously with an increasing degree of degeneration of the spermatogenic tissue. These developments are further underlined by the findings that a much more severe degeneration of the spermatogenic tissue occurred in the animals on HAO than in those on the fat-free diet (Table III).

During the following experimental period (15th through 25th week), there was practically no change in body weight of the animals fed the fat-free or the HAO diets. However, the absolute and relative weights of the testis of these animals decreased and the degenerative changes of the spermatogenic tissue increased.

The HHO had only half the amount of *trans* acids observed in HAO (Table I). The former oil, however, contained about equal amounts of *trans* $C_{18:1}$, $C_{20:1}$ and $C_{22:1}$ and smaller amounts of *trans* dienes and trienes, whereas HAO contained practically all of its *trans* acids as elaidic acid. The partially hydrogenated oils also contained varying but rather small amounts of positional isomers (6). Whether the effects observed may, in part be ascribed to either the geometric or the positional isomers, or a combination of the two types of isomeric fatty acids is the subject of further studies.

The present findings raise two questions. First, the appropriateness of talking about degeneration of the spermatogenic tissue after five weeks of feeding 28% HHO to these young animals. Maybe the degeneration is merely a dietary effect resulting in a delay in the development of the spermatogenic tissue during the period of sexual maturation. In this case, it must be concluded from the results obtained during the last 21 weeks of the experiments (Table III) that the spermatogenic tissue never did develop in these animals. Davis et al. (16) have reported that changes in lipid composition occur in the rat testes at the same time as the appearance and maturation of the spermatids. Second, during the experimental period a slight increase in the absolute and relative weights of the testis was observed in these animals (Table III). Whether this weight increase concerns the deposition of lipids or their compositional changes will be shown by analytical work which is in progress.

It should be pointed out that the relative weights of the testes of Groups 1, 2, 3 and 4 (Table III), after 26 weeks of experiment, are almost equal, although the degenerative changes of those from Groups 1 and 2 are very severe, while the testes from Groups 3 and 4 are normal.

The present experiments underline the fact that partially hydrogenated oils devoid of EFA, as the sole dietary fat of rats, stress the EFA deficiency. Further, partially hydrogenated marine oils apparently have a stronger effect in this respect than partially hydrogenated vegetable oils and fat-free diets. The reduced caloric efficiency and the tissue degenerations described can possibly be explained as membrane changes or reduced enzymatic activity, or both, due to changes in lipoprotein structures.

ACKNOWLEDGMENT

Mrs. E. Kortegaard and Miss B. Rasmussen gave technical assistance in the histological work.

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[Received November 18, 1968]

Essential Fatty Acid-Deficient Rats: II. On the Fatty Acid Composition of Partially Hydrogenated Arachis, Soybean and Herring Oils and of Normal, Refined Arachis Oil

G. HØLMER and **E. AAES-JØRGENSEN**, Danish Fat Research Institute and
Department of Biochemistry and Nutrition, Polytechnic Institute, Lyngby, Denmark

ABSTRACT

The fatty acid composition of partially hydrogenated arachis (HAO), partially hydrogenated soybean (HSO) and partially hydrogenated herring (HHO) oils and of a normal, refined arachis oil (AO) was studied in detail by means of direct gas liquid chromatography, ultraviolet and infrared spectrophotometry and by thin layer chromatography fractionation on silver nitrate-silica gel plates followed by gas liquid chromatography. It was shown that the partially hydrogenated oils all contained fatty acids with *trans* double bonds. In the plant oils, the *trans* acids were present mainly as elaidic acid. The HHO showed an almost equal distribution between *trans* 18:1 $\omega 9$, *trans* 20:1 $\omega > 9$ and *trans* 22:1 $\omega > 9$. Some *trans* configuration was also found in the C_{20} - and C_{22}-dienes and trienes of the HHO. In all the oils, conjugated fatty acids were present in minor amounts only (<0.5%). Special attention was given to the ω-acids known to be of specific nutritional value. The HSO contained about 32% linoleic acid, whereas the content of *cis,trans* + *trans,cis* and *trans,trans* octadecadienoic isomers was 1.7% and 0.5%, respectively. The amount of linoleic acid in the HSO was even higher than that of AO (29%). The HAO contained only 0.8% 18:2 $\omega 6$ (linoleic acid). Further, two 18:2 fatty acids with $\omega > 6$, a *cis,cis* and a *trans,trans* isomer, were present in small amounts. The HHO contained 0.5% 18:2 $\omega 6$ (linoleic acid). Isomers of 18:2 $\omega > 6$ were also found in the HHO. They may be hydrogenation products of higher unsaturated C_{18}-acids originally present. All the C_{20}- and C_{22}-dienes and trienes were shown to have an ω-chain greater than 6. Fatty acids with $\omega 6$-structure were not formed during partial hydrogenation of the oils studied.

INTRODUCTION

Analyses of the fatty acid composition of unhydrogenated plant or fish oils are easily performed and therefore widely reported in the literature (1-3). Partially hydrogenated oils are much more complicated and varied in fatty acid composition because of formation of positional and geometrical isomeric acids. Only a few data for such oils have been published so far (4,5). Because of the complexity of the fatty acid pattern, only trends of their structure have been demonstrated. Our nutritional experiments (6) with this type of oil made it necessary to determine, as far as possible, the fatty acid composition of partially hydrogenated arachis oil (HAO) partially hydrogenated soybean oil (HSO) and partially hydrogenated herring oil (HHO), as well as of normal refined arachis oil (AO).

MATERIALS AND METHODS

All the oils examined were commercially processed and refined oils (6). The melting points were given as follows: HAO, 41 C; HSO, 27 C; and HHO, 35-37 C.

Gas Chromatography

The methyl esters were prepared according to the *trans*-methylation method of Stoffel et al. (7). Fifty milligrams of the oil samples were methylated with a mixture of 8 ml of 5% dry HCl in superdry methanol and 1 ml of benzene. To prevent oxidation, 1 ml of a solution of 0.1% hydroquinone in dry methanol was added. The mixture was refluxed for 2 hr at 80 C on a water bath.

Thin layer chromatography (TLC) on Silica Gel G (Merck, Darmstadt, Germany) of the methyl esters in a system of petroleum ether-diethyl ether (9:1 v/v) proved complete methylation of all samples.

Gas chromatography of the methyl esters was performed on a Beckman GC 4 instrument with a FID detector using a 1/8 in. o.d. x 6 ft column packed with 15% DEGS on Chromosorb W (A W) (Applied Science Labs., Inc., State College, Pa.); column temperature was 175 C detector temperature 240 C and inlet temperature 230 C. Helium was used as carrier gas at a flow rate of 38-40 ml/min; air flow was 250 ml/min and hydrogen flow was 40 ml/min. The signals were recorded on a 1.0 mV Texas Servoriter recorder equipped with a DISC integrator. Calibration of the detector was made by means of a quantitative standard mixture (Applied Science Labs., Inc., Standard No. H 104). Direct quantitation of the integrator

TABLE I

Fatty Acid Composition of Dietary Fats Determined by Direct GLC
(w % of Total Methyl Esters)

Fatty acid	Partially hydrogenated arachis oil[a] (HAO)	Partially hydrogenated soybean oil (HSO)	Arachis oil[a] (AO)	Partially hydrogenated herring oil (HHO)
12:0	1.1	0.2	Trace	Trace
14:0	0.8	0.4	Trace	7.4
15:0 br.	---	---	---	0.8
15:0	---	---	---	0.7
16:0 br.	---	---	---	0.3
16:0	10.7	10.6	9.3	14.9
16:1	1.0	0.6	0.3	5.6
17:0	---	---	---	1.2
17:1	---	---	---	0.8
18:0	13.9	8.2	3.5	6.5
18:1	64.8	40.8	48.5	13.0
18:2 ω>6	2.5			1.6
18:2 ω6	0.8	34.8[b]	28.8[b]	1.3
18:3 ω3	---	4.2[c]	1.1	---
20:0	2.0		1.4	4.8
20:1	1.2		1.7	11.3
20:2	---	---	---	3.5
20:3	---	---	---	1.6
20:4	---	---	---	---
22:0	1.3	0.3	2.8	5.3
22:1	---	---	0.4	14.1
22:2	---	---	---	3.3
22:3	---	---	---	1.9
24:0		---	2.1	Trace
24:1	---	---	---	---
Σ saturated	29.8	19.7	19.1	41.9
Σ monoenoic	67.0	41.4	50.9	44.8
Σ polyenoic	3.3	39.0	29.9	13.2

[a]The HAO did not originate from the AO used in these experiments.

[b]Contains isomers as minor compounds.

[c]Small amounts of $C_{20:0}$ and $C_{20:1}$ are included.

strokes corresponded with weight percentages. Therefore the calculations of fatty acids were made without using correction factors.

Peak identifications were made with reference methyl esters obtained from The Hormel Institute, Austin, Minn., or, when standards were not available, by plotting carbon numbers versus log relative retention times.

Ultraviolet Spectrophotometry

The content of conjugated fatty acids was determined by the technique described in AOCS Official Methods (8). Readings for conjugated dienes and trienes were taken at 233 and 268 nm, respectively, in a Beckman DU spectrophotometer.

Infrared Analysis

All the oils were submitted to infrared analysis to determine the total content of *trans* fatty acids. A Perkin Elmer model 257 infrared spectrophotometer was used for the measurements. NaCl microcells, pathway 0.1 mm, totally containing about 25 μl, were used. Measurements were performed on 10% solutions of the methyl esters in CS_2 (Merck, Darmstadt, Germany). Elaidic acid (British Drug House, Ltd., Poole, England), recrystallized from alcohol, was converted to its methyl ester. A standard solution containing 10.71 mg ester per 100 μl was used for quantification. The presence of single *trans*-double bonds, absorption peak at 10.35 mμ (965 cm^{-1}), and of conjugated *trans* bonds, absorbing at 10.14 mμ (986 cm^{-1}), was investigated. The results are given as percentage of *trans* monoene.

Thin Layer Chromatography

The partially hydrogenated oil samples all contained a variety of positional and geometrical isomers. Therefore, a simple gas chromatographic separation of the fatty acid methyl esters of these oils gave rise to overlapping of several peaks.

TABLE II

Fatty Acid Composition of Partially Hydrogenated Arachis Oil (wt %) Determined by TLC Fractionation on Silver-nitrate Plates and Subsequent GLC

	Fatty acid	Rel. ret. time (18:0)	Per cent of fraction	Per cent of total oil
Fraction 1:	12:0	0.158	1.7	1.1
Saturated	14:0	0.288	2.1	0.8
acids	16:0	0.535	37.0	10.7
	17:0	0.721	Trace	Trace
	18:0	1.000	47.9	13.8
	20:0	1.84	5.6	2.0
	22:0	3.43	4.0	1.2
	24:0	6.37	1.8	Trace
Fraction 2:	t 16:1	0.624	1.2	0.7
Trans	t 18:1	1.15	95.4	42.1
Monoenes	t 20:1	2.02	2.4	0.6
	t 22:1	3.68	1.0	Trace
Fraction 3:	c 16:1	0.630	1.9	0.3
Cis	c 18:1	1.14	88.2	22.7
Monoenes +	c 20:1	2.07	3.7	0.6
trans, trans	c 22:1	3.86	Trace	Trace
Diene	c 24:1	6.72	Trace	Trace
	t,t 18:2 ω>6	1.37	6.1	0.9
Fraction 4:	c,c 18:2 ω>6	1.34	65.7	1.6
Cis-dienes	c,c 18:2 ω6	1.44	34.3	0.8
Per cent *trans* fatty acid (10.35 mμ)			Measured (IR)	47
			Calculated	45.2

Thin layer chromatography on silver nitrate plates is commonly used to separate methyl esters from unhydrogenated oil according to the unsaturation (9,10), and according to the position of the double bond (11). This technique was used for fractionation of the partially hydrogenated oils used in these experiments. The plates were prepared in the following way: 30 g of Silica Gel G was slurried in 60 ml of a 12.5% solution of $AgNO_3$ (Merck, Darmstadt, Germany). The slurry was spread in a 0.25 mm layer on 20 x 20 cm glass plates. These were air-dried for 40 min, then activated for 45 min at 110 C and stored in a desiccator in a dry atmosphere in the dark. For separation of methyl esters, the solvent system benzene-petroleum ether (9:1 v/v) recommended by de Vries and Jurriens (10) was used. Four fifths of the plate were uniformly loaded with 5 mg of the methyl esters to be studied. For comparison, lard methyl esters containing molecules with 0, 1 and 2 double bonds, respectively, were spotted on the last fifth of the plate. After 16 cm of migration, the bands were located by spraying with 2,7-dichlorofluoresceine (0.2% in ethanol) and examined under UV light. The bands were scraped off and eluted three times with 10 ml portions of wet diethyl ether (ether-water, 98:2). The extracts were dried over anhydrous sodium sulfate, filtered and concentrated for GLC.

RESULTS

Direct Gas Chromatography

The total content of saturated acids is much higher in the HAO and the HHO than in the HSO and the AO (Table I). Furthermore, the higher values for saturated acids in the two former oils are due to different types of acids. In the HAO, palmitic and stearic acids are the dominating saturated acids, whereas the spectrum of saturated acids in the HHO is more evenly distributed, ranging from 14:0 to 22:0.

Monoenoic acids are present in considerable amounts in all the oils examined. The C_{18}-monoenoic acid is the main component in the plant oils, whereas the partially hydrogenated marine oil contains about equal amounts of octadecenoic, eicosenoic and docosenoic acids; furthermore, palmitoleic is present. The *trans* content of these acids will be discussed later in this paper.

The HSO and the AO contained very large amounts of C_{18}-dienoic acid. However, for the HSO it remains to be verified whether this acid is linoleic acid only, or whether it contains isomers with less or no potency as essential fatty acid.

In HAO and HHO, only small values for 18:2 are seen. The main component of these 18:2 acids is identified below as an isomer of linoleic acid.

TABLE III

Fatty Acid Composition of Partially Hydrogenated Soybean Oil (wt %) Determined by TLC on Silver-nitrate Plates and Subsequent GLC

	Fatty acid	Rel. ret. time (18:0)	Per cent of fraction	Per cent of total oil
Fraction 1: Saturated acids	12:0	0.159	0.5	0.2
	14:0	0.290	1.4	0.4
	16:0	0.535	52.9	10.6
	18:0	1.000	41.0	8.2
	20:0	1.81	2.2	0.4
	22:0	3.36	1.9	0.3
	24:0	6.41	---	Trace
Fraction 2: *Trans* monoenes	t 16:1	0.615	3.2	0.6
	t 18:1	1.16	96.8	10.6
	t 20:1	2.08	---	Trace
Fraction 3: *Cis* monoene + *trans, trans* Diene	c 18:1 $\omega<9$	1.22	90.6	7.1
	c 20:1	2.08	5.2	0.3
	t,t 18:2 $\omega6$	1.44	4.2	0.5
Fraction 4: *Cis* monoene	c 16:1	0.617	---	Trace
	c 18:1 $\omega9$	1.19	100	23.0
Fraction 5: *Cis, trans* Diene (*cis* monoene)	c,t t,c 18:2 $\omega6$	1.46	85.7	1.7
	c 18:1 ($\omega>9$)	1.15	14.3	Trace
Fraction 6: *Cis, cis* diene	c,c 18:2 $\omega6$	1.46	100	31.8
Fraction 7: *Cis, cis, cis* triene	c,c,c 18:3 $\omega3$	1.94	83.6	3.4
(cis, cis diene)	c,c, 18:2 $\omega6$[a]	1.46	16.3	0.8
Per cent *trans* fatty acid (10.35 mμ)			Measured[b]	10.7
			Calculated	13.8

[a]More than one methylene group between the double bonds.

[b]In this sample small amounts of conjugated *trans* may also be present.

The HHO contained a rather high amount of total polyenoic acids. This is primarily due to C_{20}- and C_{22}-dienoic and trienoic acids. The nature of these acids will be discussed below.

Total Conjugated Fatty Acids (UV Spectrophotometry)

The HAO, HSO, AO and HHO contained 0.3%, 0.4%, 0.2% and 0.4% (weight) respectively of conjugated dienes, whereas conjugated trienes were absent. Therefore, the overlapping of conjugated acids in GLC is of minor or no importance.

Total Trans Fatty Acids (IR Spectrophotometry)

The HAO contained 47% of *trans* fatty acids. Of these more than 95% were C_{18}-monoene as deduced from GLC (Table II). In HSO, only 10.7% total *trans* fatty acids were found (Table III).

The HHO showed 22.9% *trans* fatty acids present (Table V). In contrast to the two partially hydrogenated plant oils, this oil had greater amounts of monoenes with longer chains (C_{20} and C_{22}) and also some long chain dienes and trienes, among which the *trans* bonds could be distributed (Table I). The possible occurrence of *trans* dienes in HSO and HHO will be discussed below.

Conjugated *trans* acids were not present in appreciable amounts in any of the oils examined in accordance with the direct spectrophotometric determination of total conjugated fatty acids.

TLC Argentation Chromatography of Methyl Esters Followed by GLC

Partially Hydrogenated Arachis Oil. TLC on silver nitrate plates gave four distinct bands for HAO (Table II). From these results it is evident that directly measured *trans* configuration and the calculated values are in agreement. This supports the identification procedure of the fatty acids. The main *trans* component is apparently

TABLE IV

Fatty Acid Composition of Arachis Oil (wt %)
Determined by TLC on Silver-nitrate Plates Followed by GLC

	Fatty acid	Rel. ret. time (18:0)	Per cent of fraction	Per cent of total oil
Fraction 1: Saturated acids	12:0	0.155	---	Trace
	14:0	0.288	---	Trace
	16:0	0.545	50.6	9.3
	17:0	0.721	---	Trace
	18:0	1.000	19.9	3.5
	20:0	1.84	7.1	1.4
	22:0	3.44	14.1	2.8
	24:0	6.41	8.4	2.1
Fraction 2: Monoenes	c 16:1	0.631	0.6	0.3
	c 18:1	1.19	95.4	48.5
	c 20:1	2.09	3.2	1.7
	c 22:1	3.81	0.8	0.4
Fraction 3: Diene (*cis* monoene)	c,c 18:2 ω6	1.47	99.5	28.8
	c 18:1 ω>9	1.15	0.5	Trace
Fraction 4: Triene	c,c,c 18:3 ω3	1.92	---	1.1

elaidic acid. The *trans,trans*-C_{18}-dienoic acid does not have the ω6-configuration, as deducted from the GLC retention time which indicates a longer ω-chain. A corresponding *cis, cis*-C_{18}-dienoic acid is also present. Only about 0.8% of linoleic acid (*cis* 9,*cis* 12-18:2) seems to be left in the sample after the partial hydrogenation. Palmitic and stearic acids are the dominating saturated fatty acids of HAO.

Partially Hydrogenated Soybean Oil. In this oil, argentation chromatography gave seven bands which were analyzed (Table III).

The relatively small amounts of total *trans* fatty acids in HSO are to a great extent due to monoenoic acids, primarily elaidic acid. By the highly selective hydrogenation technique used for this oil, *trans* isomers of dienoic acid were not formed. Further, the content of trienoic acid is low, 3.4%, and it seems to be present as linolenic acid only.

The fact that the amount of calculated *trans* acids is higher than the actually measured amount could throw the identification of the *trans*-diene isomers into doubt. However, it appears more reasonable to suggest that the experimental deviations in the IR determination and in the TLC fractionation may account for the difference because of the small amounts present.

Arachis Oil. Fractionation of the unhydrogenated arachis oil by TLC on silver nitrate plates showed that no positional isomers were present.

Four different zones were separated (Table IV) and the methyl esters recovered.

Monoenoic acids are nearly exclusively represented by oleic acid, which constitutes half of all the fatty acids of the arachis oil.

Linoleic acid is recovered in a band together with very small amounts of an isomer of a C_{18}-monoenoic acid. The C_{18}-dienoic ω6-acid comprised 28% of the fatty acids and is the only polyenoic acid found, apart from a minor amount of linolenic acid.

Partially Hydrogenated Herring Oil. Partial hydrogenation of highly unsaturated fish oils gives rise to a variety of isomers, geometric as well as positional. An attempt to account for all constituents was not tried. Fractionation on $AgNO_3$-plates revealed eight distinct bands which were analyzed by GLC (Table V). However, fractions 7 and 8 contained very small amounts of fatty acids, which were detectable only as traces in the original oil. The efforts were concentrated on identifying geometric and positional isomers together with *cis*-ω-6-acids.

For this oil sample (HHO), the large number of isomers made it more convenient to calculate the area percentage of peaks with the same retention time instead of using percentage of fraction. This method of calculation thus gives the percentage of total area response in GLC for each component with almost the same retention time.

A tentative identification was made by means of TLC and GLC. On silver nitrate plates, *trans* fatty acids will migrate further than the corresponding *cis* acids, but in GLC the retention times will be nearly equal. For positional isomers, both the ω-chain and the number of methylene groups between double bonds influence the TLC migration (10,11).

TABLE V

Fatty Acid Composition of Partially Hydrogenated Herring Oil (wt %) Determined by TLC on Silver-nitrate Plates Followed by GLC

	Fatty acid	Rel. ret. time (18:0)	Percentage of peak with same ret. time	Per cent of total oil
Fraction 1: Saturated acids	12:0	0.145	---	Trace
	14:0	0.290	---	7.4
	15:0 br.	0.321	---	0.8
	15:0	0.381	---	0.7
	16:0 br.	0.465	---	0.3
	16:0	0.535	---	14.9
	17:0	0.712	---	1.2
	18:0	1.000	---	6.5
	20:0	1.86	---	4.8
	22:0	3.50	---	5.3
	24:0	6.35	---	Trace
Fraction 2: *Trans* monoenes + small amounts of lower saturated acids	14:0	0.288	---	---
	16:0	0.530	---	---
	18:0	1.000	---	---
	t 18:1 ω9	1.16	8.6	1.1
	t 20:1 ω>9	2.09	36.8	4.2
	t 22:1 ω>9	3.84	40.1	5.7
Fraction 3: *Trans* monoenes	t 16:1 ω7	0.634	21.2	1.2
	t 18:1 ω9	1.16	30.8	4.0
	t 20:1 ω>9	2.06	11.9	1.3
	t 22:1 ω>9	3.80	4.6	0.6
	t 24:1 ω>9	7.07	100	Trace
Fraction 4: *Cis* monoenes	c 16:1 (ω7)	0.630	27.8	1.6
	c 17:1 (ω8)	0.850	36.4	0.3
	c 18:1 ω9	1.15	8.5	1.1
	c 18:1 ω<9	1.24	3.6	0.5
	c 20:1 ω>9	2.14	39.0	4.4
	c 22:1 (ω>9)	3.84	52.5	7.4
Fraction 5: *Cis* monoenes, *Cis, trans* dienes, *Cis, trans, trans* triene	c 16:1 (ω7)	0.639	51.0	2.8
	c 17:1 (ω8)	0.855	63.6	0.5
	c 18:1 ω9	1.16	48.6	6.3
	c,t 18:2 ω>6	1.36	39.8	0.6
	c,t 18:2 ω6	1.47	9.7	0.1
	c 20:1 ω>9	2.07	12.3	1.4
	c,t 20:2 ω>6	2.46	37.4	1.3
	c,t,t 20:3 ω>6	2.76	13.3	0.2
	c 22:1 ω>9	3.79	2.7	0.4
	c,t 22:2 ω>6	4.46	39.1	1.3
Fraction 6: *Cis,cis* dienes, *Cis,cis,trans* trienes	c,c 18:2 ω>6	1.36	50.8	0.8
	c,c 18:2 ω6	1.47	48.0	0.6
	c,c 20:2 ω>6	2.44	55.5	2.0
	c,c,t 20:3 ω>6	2.68	55.9	0.9
	c,c 22:2 ω>6	4.41	57.0	1.9
	c,c,t 22:3 ω>6	4.97	53.9	1.0
Fraction 7: *Cis,cis* dienes, *Cis,cis,cis* trienes	c,c 18:2 ω>6[a]	1.36	9.4	0.2
	c,c 18:2 ω6[a]	1.47	42.3	0.5
	c,c 20:2 ω>6[a]	2.40	7.2	0.2
	c,c,c 20:3 ω>6	2.72	30.8	0.5
	c,c 22:2 ω>6[a]	4.30	3.9	0.1
	c,c,c 22:3 ω>6	5,11	46.1	0.9
Fraction 8:	Trienes and tetraenes with ω>6			Trace
Per cent *trans* fatty acid (10.35 mμ)			Measured	22.9
			Calculated	24.1

[a]These acids may have a methylene interruption between double bonds different from the similar acids in Fraction 6.

However, Morris et al. (11), studying the separation of isomers, claim that benzene-petroleum ether (80:20) at room temperature, and one development, does not separate the isomers completely. In the present work only one development was used. Therefore, the separation according to positional isomerism probably is not complete. GLC of fatty acids with different ω-chains would give rise to different retention times.

Figure 1 shows the plot of log relative retention time versus carbon number for fatty acids of various TLC fractions of HHO. From the plots it can be deduced whether or not the longer chain dienoic and trienoic fatty acids have the ω6 configuration. It was found that none of the dienes or trienes formed during the partial hydrogenation were fatty acids with ω6 configuration. Comparison of retention times for the 20:3 and 22:3 from partially hydrogenated herring oil, with retention times for 20:3 ω9 and 22:3 ω9 found in tissues of fat-deficient rats, indicates an ω-carbon chain of about 9 carbon atoms; in the same way it was recognized that the C_{20}- and C_{22}-monoenoic acids had an ω-chain greater than 9. The ω-chains in C_{16}-monoenoic and C_{17}-monoenoic acids were shorter than 9, they apparently were the original ω7 and ω8, respectively.

The total amount of EFA in HHO is 0.6% *cis,cis*-18:2 ω6 (Table V). Trace amounts of *cis,trans* 18:2 ω6 will not influence the EFA deficiency syndromes, as this type of fatty acid is questionable as EFA (12). *Trans,trans*-linoleic acid apparently is not present.

The total content of *trans* fatty acids amounted to 24.1% in HHO, which fits fairly well with the directly measured 22.9% (Table V). Correction for non-additive appearance of *trans*-double bonds in dienes (4,5) were not made. This would give even better agreement with the direct measurement.

The distribution of corresponding *cis* and *trans* fatty acids is also seen in Table V. Geometric isomers show the same relative retention time in GLC, therefore the total percentage of any *trans* isomer can be seen in, or calculated from, the Table. Thus, the content of *cis,trans*-20:2 ω>6 is 37% of the total 20:2 ω>6. The *cis,cis* isomer accounts for about 55%, whereas the remaining 7% probably is a positional isomer with more than one methylene group interrupting the double bonds. The docosadienoic acid shows about the same proportions between its isomers.

The trienoic acids 20:3 ω>6 and 22:3 ω>6 are also present as isomers. *Cis,trans,trans* 20:3 ω>6, *cis,cis,trans* 20:3 ω>6 and all-*cis* 20:3

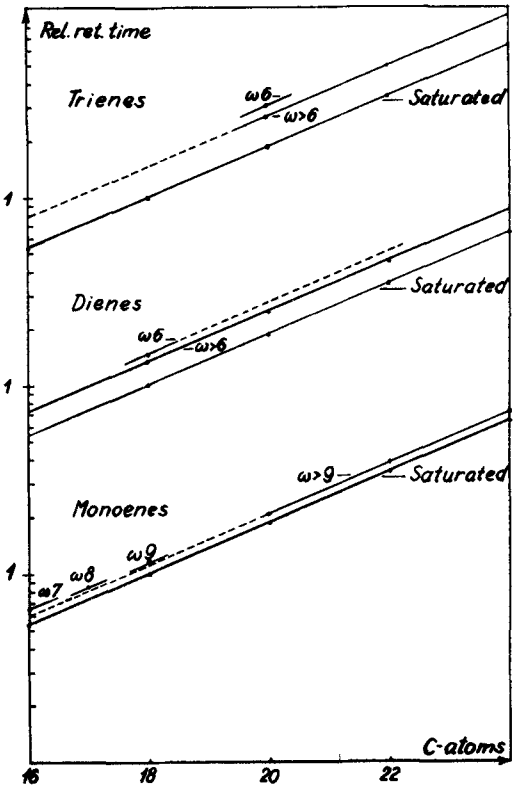

FIG. 1. Identification of fatty acid isomers in fractions of partially hydrogenated herring oil.

ω>6 constituted 13%, 56% and 31% of the total 20:3 ω>6, respectively, whereas the all-*trans* isomer was absent. For 22:3 ω>6, no all-*trans* nor *cis,trans,trans* isomers were recognized, whereas the *cis,cis,trans* and the all-*cis* isomers were present in about equal amounts.

DISCUSSION

The main differences in fatty acid distribution of the dietary oils were pointed out in Table I. Neither geometric nor positional isomers were discussed in this connection. However, the $AgNO_3$ fractionation made it possible to make comparisons of the isomers of the different dietary oils. In the plant oils, the occurrence of ω6 acids, i.e., essential fatty acids, is only possible among the C_{18}-acids, whereas the HHO has both C_{20} and C_{22}-acids with two or three unsaturated bonds.

The HAO contained 0.8% 18:2 ω6. This is about the same amount as found in HHO. However, this latter oil also contained 0.5% of a *cis,cis* 18:2 isomer with an ω6 carbon chain, but probably with more than one methylene

group interrupting the double bonds. This finding is in clear contrast to the very high amount of linoleic acid, 28% in AO.

The HSO, which could be expected to contain high amounts of geometrical isomers of 18:2, contained about 32% linoleic acid, only 1.7% *cis,trans* isomers, and even less *trans,trans* isomers, namely 0.5%.

None of the isomers formed during partial hydrogenation of the higher unsaturated fatty acids originally present in the herring oil belonged to the ω6 family. All of the acids identified showed ω-carbon chains greater than 6.

From the TLC fractionations and the GLC identifications it can be said that nearly all the *trans* content in the HAO is present as elaidic acid. This is also the case for HSO; however, it was shown that *cis,trans* and *trans,trans* isomers of linoleic acid are present in smaller amounts.

In the HHO, *trans* bonds are present in many different fatty acids. About 18% of the total fatty acids are monoene *trans* fatty acids distributed equally among *trans* 18:1 ω9 (elaidic acid), *trans* 20:1 ω>9 and 22:1 ω>9. The rest of the *trans* double bonds (6%) are distributed in seven different components, among which are *cis,trans* 20:2 ω>6 and 22:2 ω>6 as well as *cis,cis,trans* 20:3 ω>6 and 22:3 ω>6 acids.

ACKNOWLEDGMENT

Technical assistance in the analytical work was rendered by Miss Britta Offenberg.

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[Received November 18, 1968]

Essential Fatty Acid-Deficient Rats: III. Distribution of Lipid Classes in Rat Testes After Feeding Partially Hydrogenated Oils

G. HØLMER and E. AAES-JØRGENSEN, Danish Fat Research Institute
and Department of Biochemistry and Nutrition and Polytechnic Institute, Lyngby, Denmark

ABSTRACT

Total and relative amounts of neutral lipids (NL) and phospholipids (PL) as well as the distribution of various lipid classes in these were determined in testes of rats fed different types of partially hydrogenated oils for 5,15 and 26 weeks. The dietary fats were partially hydrogenated arachis oil (HAO), partially hydrogenated soybean oil (HSO), partially hydrogenated herring oil (HHO) and, for comparison, arachis oil (AO). An additional series of animals was reared on a fat-free diet throughout the entire experiment. The total amount of NL is decreased by EFA deficiency parallel with the development of the degenerative changes of the spermatogenic tissue. The relative amounts of NL in the testis are not influenced by EFA deficiency during the first stages of degeneration. However, feeding of HHO for 26 weeks resulted in a marked decrease in NL. The total content of PL is directly related to tissue degeneration. This observation is supported by the data obtained after 5 weeks of feeding HHO and by the correspondence between the results found after 15 and 26 weeks on HAO and the fat-free diets, respectively. The relative amount of PL is less influenced by EFA deficiency, but severe degenerations as found for the group fed HHO are followed by decreases. The neutral lipids had three main fractions: triglycerides (TG), free fatty acids (FFA) and cholesterol (Chol). FFA was found to be the main fraction of NL after 5 weeks, whereas TG was the main component of NL after 15 and 26 weeks, especially in the animals with degenerated testes. The presence of the large quantities of FFA is discussed. Cholesterol was decreased markedly in the EFA deficient rats fed partially hydrogenated oils, but not in the fat-free reared groups. The variations in the PL distribution during the experiment were small with regard to the two main PL classes, the phosphatidylcholines and the phosphatidylethanolamines. The most remarkable change among the PL classes was an increase in the percentage of sphingomyelins when the spermatogenic degenerations developed.

INTRODUCTION

In a previous paper (1) were reported the growth, gross symptoms and histological changes in the testes and epididymides of rats fed partially hydrogenated arachis oil (HAO), partially hydrogenated soybean oil (HSO) and partially hydrogenated herring oil (HHO). Animals fed arachis oil (AO) were used as controls. The fatty acid pattern of the above mentioned oils, which were used as the sole dietary fat, has been studied in detail (2). Large individual differences were found in the fatty acid composition of the dietary fats.

Previous studies in this field have been made by Bieri and Prival (3), who described some of the lipid classes of testes from normal animals of different species. Holman and Hofstetter (4) studied the fatty acid composition of the lipids from bovine and porcine reproductive tissues. In the present paper we are reporting on the distribution of the various lipid classes from the testes of rats fed diets containing various partially hydrogenated oils, arachis oil or a fat-free diet.

EXPERIMENTAL PROCEDURES

The details of the animal experiment were described earlier (1). The dietary fat comprised 28% (by weight) of the total basal diet. After the animals were killed, both testes were removed immediately, weighed and frozen. One testis from each animal was used for histology (1) and the other for lipid analysis. In order to get enough material for all the analytical examinations it was decided to make two pools per group, each consisting of four testes. This was especially necessary in the cases of severely degenerated testes.

Homogenization and extraction were made simultaneously in a MSE homogenizer with $CHCl_3$:CH_3OH (2:1 v/v), according to Folch et al. (5). The purified extract was chromatographed on a column (2.6 cm i.d., length 10 cm), containing a mixture of 15 g silicic acid (Baker Analyzed Reagent, Deventer, Holland)

TABLE I

Total and Relative Weight of Neutral Lipids and Phospholipids of the Right Testes of the Rats[a]

Group no.	1	2	3	4	5
Diet characteristics[b]	Fat-free	28% HAO	28% HSO	28% AO	28% HHO
After 5 weeks on experimental diets					
NL total (mg)	50.3 ± 1.1	51.1 ± 2.1	65.1 ± 1.6	62.3 ± 0.9	27.5 ± 0.0
NL relative (mg/g)	9.3 ± 0.1	11.0 ± 0.5	10.4 ± 0.3	10.3 ± 0.1	12.3 ± 0.1
PL total (mg)	65.5 ± 2.5	48.3 ± 0.7	63.3 ± 0.3	64.3 ± 1.6	18.6 ± 0.8
PL relative (mg/g)	12.2 ± 0.3	10.4 ± 0.1	10.1 ± 0.0	10.6 ± 0.3	8.3 ± 0.4
After 15 weeks on experimental diets					
NL total (mg)	36.7 ± 4.3	30.2 ± 2.5	72.0 ± 6.6	77.4 ± 7.0	29.3 ± 0.9
NL relative (mg/g)	10.1 ± 0.8	10.3 ± 0.8	11.9 ± 0.9	12.5 ± 1.1	13.9 ± 0.1
PL total (mg)	42.3 ± 4.3	31.0 ± 0.7	75.4 ± 5.2	75.7 ± 4.2	17.4 ± 0.1
PL relative (mg/g)	11.6 ± 0.8	10.6 ± 0.3	12.4 ± 1.0	12.2 ± 0.7	8.2 ± 0.2
After 26 weeks on experimental diets					
NL total (mg)	29.8 ± 2.4	33.8 ± 0.0	81.4 ± 7.0	82.3 ± 0.7	27.0 ± 1.8
NL relative (mg/g)	9.6 ± 0.8	10.6 ± 0.0	10.6 ± 0.9	11.8 ± 0.0	7.7 ± 0.4
PL total (mg)	28.5 ± 2.0	30.3 ± 0.2	87.0 ± 0.5	85.9 ± 1.8	27.9 ± [c]
PL relative (mg/g)	9.2 ± 0.6	9.5 ± 0.1	11.3 ± 0.0	12.3 ± 0.2	7.8 ± [c]

[a]Standard deviations for two pools of four testes each are given.

[b]HAO, partially hydrogenated arachis oil; HSO, partially hydrogenated soybean oil; AO, Arachis oil; and HHO, partially hydrogenated herring oil.

[c]Only one quantitative determination.

and 5 g Hyflo supercel (Johns-Manville Co., New York), prewashed thoroughly with chloroform.

The neutral lipids (nonphospholipids, NL) were eluted with 150 ml of chloroform and the phospholipids (PL) with 150 ml of methanol. These two main fractions were concentrated on a rotating vacuum evaporator (Buchi, Switzerland) at a temperature of 40 C. Suitable aliquots were taken for quantitation by weight.

Lipid classes of the NL fraction were separated by TLC on Silica Gel G (Merck, Darmstadt, Germany) in a solvent system containing redistilled petroleum ether (bp 60-70 C, Chr. F. Petri, Copenhagen), diethyl ether (Ph.Nord., Chr. F. Petri, Copenhagen), and acetic acid (Merck, Darmstadt, Germany) in the ratio 70:30:1 (v/v/v).

Cholesterol and cholesterol esters were determined by the Liebermann-Burchard reaction as modified by Sperry and Webb (6). The scrapings from the TLC plate containing cholesterol and cholesterol esters were used directly for the measurements. Blank areas from the plates showed no color reaction. Cholesterol, purified via the dibromide (7) and cholesterol palmitate, purified by TLC, were used as standards. The triglycerides and the free fatty acids were determined by weighing. Elution from the Silica Gel G was made with three portions of wet diethyl ether, ether-water (98:2).

The PL fraction was separated into classes by TLC on Silica Gel H (Merck, Darmstadt) suspended in 1 mM Na_2CO_3, according to Skipski (8).

The separation of testis phospholipids was performed in a solvent system of chloroform-methanol-water (65:25:4 v/v/v) according to Wagner et al. (9). In this system, the phospholipids present interfered less with each other than in other systems tried. The only disadvantage is that phosphatidylserine (PS) is not completely resolved from phosphatidylcholine (PC). However, the PS fraction was found to be a minor fraction (3-5%) in testis phospholipids compared to PC. This is in accordance with the findings of Bieri and Andrews (10) and Davis et al. (11).

The TLC was performed in a saturated chamber, migration 15-16 cm. Spots were visualized with iodine vapor. After disappearance of the iodine color, the fractions were scraped off and phosphate determined according to Bartlett (12). It was found that direct measurement of phosphate in the TLC scrapings gave reproducible results, when the plates were prewashed by developing them in the solvent system used for PL separation. Reactivation of the plate was necessary before spotting the phospholipid mixture. The analytical procedure was checked by PC and PE standards.

RESULTS AND DISCUSSION

Neutral Lipids

Total and relative amounts of NL in a pool of four testes are given in Table I.

TABLE II

Distribution of Neutral Lipid Fractions From Rat Testes as Per Cent of Total Neutral Lipids[a]

Group no.	1	2	3	4	5
Diet characteristics[b]	Fat-free	28% HAO	28% HSO	28% AO	28% HHO
After 5 weeks on experimental diets					
Cholesterol	15.6 ± 0.1	15.7 ± 2.2	12.6 ± 0.0	14.0 ± 0.4	11.9 ± 2.1
Free fatty acids	51.4 ± 0.1	57.2 ± 1.0	56.4 ± 0.1	56.0 ± 0.4	51.2 ± 0.7
Triglycerides[c]	31.9 ± 0.1	25.9 ± 1.4	30.1 ± 0.2	28.8 ± 0.2	35.2 ± 2.9
Cholesterol esters[d]	1.0 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	1.1 ± 0.2	1.4 ± 0.1
After 15 weeks on experimental diets					
Cholesterol	18.6 ± 1.0	12.0 ± 0.1	16.3 ± 1.8	11.8 ± 0.3	11.0 ± 0.3
Free fatty acids	35.1 ± 3.4	36.1 ± 3.6	41.2 ± 3.7	29.5 ± 1.4	29.3 ± 1.3
Triglycerides[c]	44.4 ± 3.4	50.9 ± 3.8	41.4 ± 5.2	57.9 ± 1.4	58.7 ± 1.7
Cholesterol esters[d]	1.6 ± 0.3	1.0 ± 0.1	1.1 ± 0.2	0.9 ± 0.1	1.1 ± 0.1
After 26 weeks on experimental diets					
Cholesterol	15.8 ± 0.7	8.3 ± 3.4	16.3 ± 0.5	12.1 ± 1.1	7.9 ± 1.0
Free fatty acids	27.6 ± 3.4	35.2 ± 7.9	46.0 ± 1.7	37.7 ± 1.7	30.4 ± 9.0
Triglycerides[c]	54.7 ± 4.6	55.6 ± 4.0	36.3 ± 1.2	49.3 ± 2.8	60.2 ± 7.7
Cholesterol esters[d]	1.9 ± 0.5	0.9 ± 0.4	1.4 ± 0.1	0.9 ± 0.1	1.4 ± 0.2

[a]Standard deviation for two pools of four testes each are given.

[b]For explanation of abbreviations, see Table I.

[c]This fraction also includes diacylglyceryl ethers. Neutral plasmalogens were only found in trace amounts, deduced by GLC.

[d]Cholesterol esters plus small amounts of nonidentified, nonpolar lipids.

Already after five weeks of experimental feeding, it is evident that the animals fed HHO are very low in total NL compared to those receiving EFA (Groups 3 and 4). However, the relative amount of NL in the HHO group seems to be even higher than for the animals receiving EFA. A suggestive explanation of this observation may be that the HHO animals have very little, if any, visible depot fat, i.e., all dietary fat is, generally speaking, metabolized. However, some triglycerides are still present in the very small testes resulting in a high relative value. This NL may, in part, be structural NL, but may be, in part, a result of accumulated NL because of slow transfer to PL of the rather unusual fatty acids present in the partially hydrogenated herring oil (2). A somewhat similar tendency is observed in the animals fed HAO (Table I). The relative weight of NL was lowest for the fat-free groups. This may be due to mobilization of NL for PL synthesis.

After 15 weeks of experiment, a remarkable decrease in total NL was observed in the rats on the fat-free diet or with 28% HAO (Table I). Simultaneously, the absolute weight of the testes, especially in the HAO group, decreased markedly.

After 26 weeks on the experimental diets, the absolute amount of NL in the testes was at the same level and very low in all three EFA-deficient groups of animals (Table I). The relative amount of NL was very low in the animals fed HHO or a fat-free diet. The absolute weight of the testes from the HHO animals was increasing from the 5th through the 25th week of experiment. The data (Table I) clearly indicate that this increase in absolute weight is not due to increased deposition of NL. The histological studies of the left testis from all of the animals (1) showed severe degenerative changes of the spermatogenic tissue after a short feeding period with HHO. A similar picture was observed later with the HAO diet, and finally with the fat-free diet. It may thus be concluded that the relative amount of NL in the testes is not necessarily affected to any marked degree by EFA deficiency, whereas the total amount of NL and the weight of the testes give valuable information about the functional status of the spermatogenic tissue. Whether the fatty acid profile of the NL compounds may further enlarge our understanding of these problems will be the subject of forthcoming papers.

Phospholipids

Values for total and relative amounts of phospholipids in testes of rats fed different dietary fats for 5, 15 and 26 weeks, respectively, are shown in Table I.

Five weeks of feeding HHO caused a very drastic decrease in the total as well as in the relative content of PL in the testes. Feeding of HAO reduced the total content of PL in the testes to some extent, whereas the relative

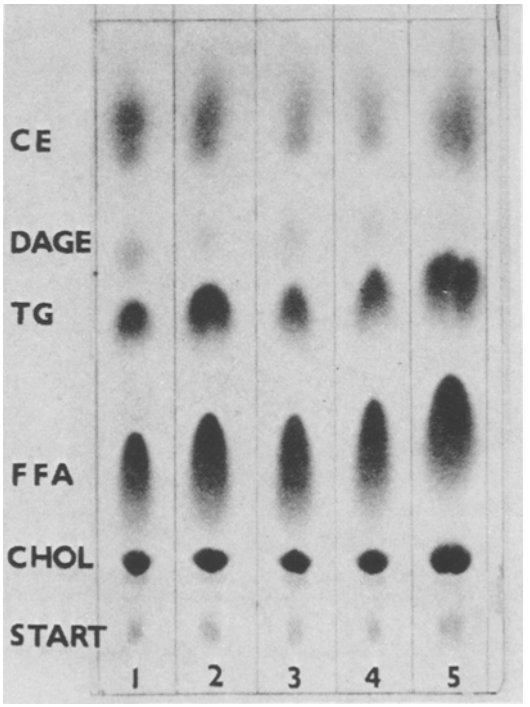

FIG. 1. Distribution of neutral lipids in testes from rats after five weeks of experimental feeding. CE, cholesterol esters; DAGE, diacylglycerol ethers; TG, triglycerides; FFA, free fatty acids; CHOL, cholesterol. The figures correspond to the various group numbers in the tables.

amount of PL was not affected (Table I). This rather short experimental period did not influence the content of PL in the testes of the fat-free reared animals, but the relative amount was increased even compared to the EFA-supplemented animals.

Fifteen weeks of experiment caused a very pronounced decrease in the absolute content of PL compared to the results after 5 weeks in the rats fed HAO and the fat-free diet, respectively. This decrease in PL is paralleled by a pronounced decrease in the weight of the testes in these groups.

After 26 weeks of experimental feeding, a further decrease was noticed in the total amount of PL in the testes of the animals fed a fat-free or a 28% HAO diet (Table I). The relative amounts of PL were also reduced.

It should be noticed that after 26 weeks the total amount of PL increased somewhat when the HHO diet was used. This is also indicated by an increase in the weight of the testes. However, the absolute and the relative amounts of PL in all of the deficient groups are now of the same order and significantly lower than in the EFA-supplemented groups.

The present data thus indicate that degeneration of the testes tissue caused by EFA deficiency (1) can be stressed by the type of dietary fat. It is paralleled by decrease amounts of total PL and NL. The relation between these facts seems well established, especially by the findings after 5 weeks of feeding HHO and the correspondence between the developments after 15 and 26 weeks on HAO and the fat-free diet, respectively. The relative amount of PL is decreased in severely degenerated animals. However, the relative content of NL does not parallel the increase in degeneration.

Distribution of Neutral Lipid Classes in Rat Testes

The results of fractionation of the NL into lipid classes are given in Table II and Figure 1.

In qualitative TLC, a distinct spot of diacylglyceryl ethers (DAGE) was seen in all groups throughout the experiment. In preparative work, i.e., quantitation of lipid classes and preparation of methyl esters for GLC, this fraction was not completely resolved and therefore was taken together with the triglycerides (TG).

Five weeks of experimental feeding gave nearly the same NL distribution in all groups examined (Table II). Surprisingly enough, the main component in the NL fraction was free fatty acids, which represented more than half of the neutral lipids.

A large amount of free fatty acids has previously been found in milt from herring. Here, 13-14% of the total milt oil were free fatty acids (13). Holman and Hofstetter (4) reported 10% free fatty acids in the total lipids of beef testes, whereas the content in pork was only 0.5%. Therefore, it is possible that the amount of free fatty acids present in the testis NL fraction may play a role in the male reproductive organs of some species.

After 15 weeks of experiment, the distribution of lipid classes in the NL fraction was different from that observed after 5 weeks. Now, the main component was the triglycerides, except for the rats on HSO, where nearly equal amounts of triglycerides and free fatty acids were found. Simultaneously, the amount of free fatty acids decreased considerably.

Extension of the experiment to 26 weeks further underlines the changed distribution of the main components of NL observed already after 15 weeks. The triglycerides are now the dominant component, except in the HSO group. Further, the cholesterol content in the HAO and HHO groups is significantly lower than that of the EFA-supplemented groups.

The reversal of the proportion of free fatty acids and triglycerides during the 26 weeks of

experiment could be the result of tissue changes related to physiological circumstances in connection with sexual maturity. However, the findings of Davis et al. (11) on changes in lipid composition of the maturing rat testis are not in agreement with our findings. They did not find free fatty acids at all in the testes. Davis et al. fed rats a Purina laboratory chow (fat per cent is not given), whereas our diets contained 28% of specific oils in a semisynthetic basal diet. Thus, these dietary differences may influence the results substantially. Oshima and Carpenter (14) used commercial rat pellets containing about 5.5% crude lipid, which was rather high in linoleic acid and contained cholesterol. They found free cholesterol and triglycerides to be the predominant classes of NL of prepubertal and adult rat testes. Only trace amounts were found of materials with R_f values corresponding to free fatty acids and free aldehydes. They stress that in order to avoid possible contamination of testes lipid with adipose tissue the tunica albuginea of the testis was removed. This coat was not removed in our experiments, but the epididymides and its fat pad was removed very carefully. We feel that discrepancies between their data and ours are not related to this latter fact.

In the present experiments: (a) only very small values were found for diglycerides and monoglycerides; (b) lysophospholipids, were not present in appreciable amounts; (c) free fatty acids in great amounts occurred in the NL fraction from all three experimental periods; and (d) storage of NL extracts for several months at -20 C did not increase the free fatty acid content markedly. The presence of the great amounts of free fatty acids have also led to the idea that they could be artifact, i.e., they were liberated during storage or analytical procedures. Davis et al. (11) and Oshima and Carpenter (14) extracted the testes immediately after killing the animals. We have stored the testes at -20 C for several months before extraction and analysis, because of the large number of animals killed at various times during a maximum experimental period of 26 weeks. Therefore, we cannot exclude the long storage as a partial explanation of the high values found for free fatty acids, although we did not find any intermediates, e.g., mono- and diglycerides or lysophosphatides.

Distribution of Phospholipid Classes in Rat Testes

Distribution of the total PL fraction from column chromatography gave seven separate phospholipid classes (Fig. 2). The results of these studies are given as percentage lipid phosphorus (Table III).

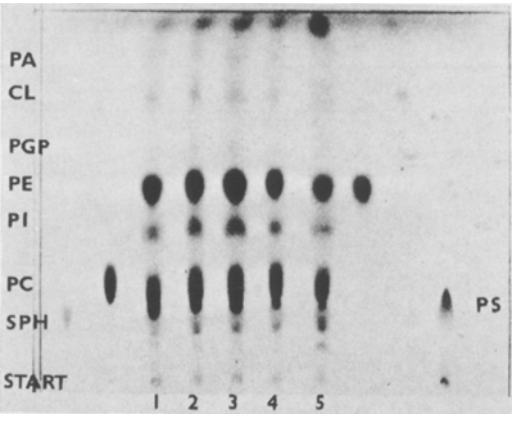

FIG. 2. Distribution of phospholipids in rat testes after five weeks of experimental feeding. CL, cardiolipin; PA, phosphatidic acid; PE, phosphatidylethanolamines; PGP, polyglycerophosphatidic acid; PI, phosphatidylinositols; PC, phosphatidylcholines; SPH, sphingomyelins; PS, phosphatidylserines. The figures correspond to the various group numbers, the remaining samples were standards.

After five weeks of experimental feeding, it was seen that the group fed HHO had an extremely high amount of sphingomyelin (SPHM) compared to all the other groups. In the latter groups, minor differences only were observed in SPHM. In the HHO group, the high value for SPHM resulted in somewhat lower values for PE.

The relative amount of SPHM (mg/g tissue) in the HHO group was 1.2 (Tables I and III), whereas the values for the other groups ranged from 0.7 to 0.9. This indicates a real increase in the SPHM in the testes of HHO animals.

The results obtained after 15 weeks of experiment (Table III) showed a pronounced increase of the SPHM content in the testes from the rats fed HAO and the fat-free diet, compared to the data after 5 weeks. In these cases the increase seems primarily to be compensated by decreases in the minor PL constituents. The relative content of SPHM in these two groups of animals was 1.2 (mg/g tissue). This is in accordance with an increasing degree of degeneration of the spermatogenic tissue (1) and similar to the picture seen in the HHO rats already after five weeks. After 15 weeks, the SPHM content was 1.6 mg/g tissue for these latter animals.

After 26 weeks on the experimental diets, the SPHM fraction increased further in the groups on HAO and the fat-free diet. Thus, at this stage, with total degeneration of the

TABLE III

Distribution of Phospholipids (Weight Per Cent of Phosphorus) in Testes of Rats

Group no.	1	2	3	4	5
Diet characteristics[a]	Fat-free	28% HAO	28% HSO	28% AO	28% HHO
After 5 weeks on experimental diets					
SPHM[d]	5.7 ± 1.1[b]	8.4 ± 0.4	7.7 ± 0.7	6.2 ± 0.1	15.2 ± 2.1
PC + (PS)	51.2 ± 0.6	49.5 ± 2.1	46.5 ± 0.5	49.8 ± 0.8	45.6 ± 1.2
PI	2.8 ± 0.5	4.1 ± 0.2	3.5 ± 0.5	4.7 ± 0.2	4.1 ± 0.0
PE + PE-plasmalogen	33.4 ± 2.1	33.9 ± 1.4	38.3 ± 1.0	34.1 ± 0.0	29.8 ± 1.1
PGP	3.6 ± 0.3	2.0 ± 0.1	1.6 ± 1.5	2.5 ± 0.1	3.0 ± 0.1
CL	3.1 ± 0.5	2.1 ± 0.1	2.6 ± 0.1	2.0 ± 0.4	1.7 ± 0.0
PA	0.4 ± 0.4	Trace	Trace	1.1 ± 1.0	Trace
After 15 weeks on experimental diet					
SPHM	10.5 ± 1.5	11.0 ± 1.0	8.5 ± 0.5	9.0 ± 1.0	19.0 ± 3.0
PC + (PS)	54.8 ± 0.8	51.5 ± 1.5	50.0 ± 4.0	54.0 ± 1.0	43.5 ± 4.5
PI	0.4 ± 0.3	Trace	2.5 ± 0.5	Trace	Trace
PE + PE-plasmalogen	32.9 ± 0.9	33.0 ± 3.0	36.5 ± 2.5	35.0 ± 0.0	34.0 ± 1.0
PGP	Trace	Trace	Trace	Trace	Trace
CL	1.3 ± 0.6	1.5 ± 0.5	2.0 ± 0.0	2.0 ± 0.0	3.5 ± 0.5
PA	0	2[c]	0.5 ± 0.5	0	0
After 26 weeks on experimental diet					
SPHM	13.5 ± 0.5	17.0 ± 1.0	9.0 ± 0.0	8.5 ± 0.5	15.8 ± 0.5
PC + (PS)	51.5 ± 2.5	48.0 ± 1.0	49.0 ± 0.0	51.0 ± 1.0	45.5 ± 1.1
PI	0	Trace	2.5 ± 0.5	2.5 ± 0.5	3.0 ± 0.3
PE + PE-plasmalogen	32.0 ± 4.0	32.0 ± 2.0	34.5 ± 0.5	33.0 ± 1.0	34.4 ± 0.0
PGP	Trace	Trace	3.0 ± 0.0	2.5 ± 0.5	Trace
CL	3.0 ± 1.0	3.0 ± 0.0	2.0 ± 0.0	2.5 ± 0.5	1.4 ± 0.0
PA	0	Trace	0	Trace	0

[a]For explanation of abbreviations, see Table I.

[b]Standard deviations for two pools of four testes each.

[c]Only one determination.

[d]SPHM, sphingomyelin; PC + (PS), phosphatidylcholine + phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PGP, polyglycerophosphatide; CL, cardiolipin; and PA, phosphatidic acid.

spermatogenic tissue (1), the SPHM values (expressed as wt % P) were about the same in all the EFA-deficient animals and significantly higher than in the EFA-supplemented animals. It should be noticed also that, throughout the experiment, the changes (calculated as per cent of phosphorus) were very small in the two major PL groups, PL and PE. Bieri and Andrews (10) found a similar relative increase in SPHM content when they compared normal and vitamin E-deficient rat testes.

In the EFA-deficient animals, the mg SPHM/g tissue was 1.2, 1.6 and 1.2 for the fat-free, the HAO and the HHO fed rats, respectively; thus indicating a real increase in SPHM with increasing degeneration of the spermatogenic tissue. The value of 1.2 for the HHO testes, which were degenerated already at an early stage of the experiment, may be related to increasing amounts of connective tissue resulting in a small increase in absolute weight of these testes (1).

The changes in the amounts of the minor phosphatides were less clear cut, but it should be mentioned that long time feeding with EFA-deficient diets decreased the content of polyglycerophosphatidic acid (PGP) and perhaps also of phosphatidylinositols (PI), whereas cardiolipin (CL) was always present in small amounts only.

From the distribution of PL (Table III) and the total amounts of PL (Table I) it appears that EFA deficiency decreased PC and PE, known as structural elements of membranes, more severely than the SPHM. A similar comparison of the relative amounts (mg/g tissue) of PC and PE revealed a slight decrease, whereas SPHM increased somewhat. The explanation of this difference may be that the pathways followed in biosynthesis of SPHM are different from those followed in the synthesis of glycerophospholipids and, apparently, less EFA-dependent. This may be due to the fatty acid composition of the SPHM, which is much less unsaturated than that of the glycerophosphatides. Feeding of isomers, geometrical as well as positional, as found in partially hydrogenated oils, may greatly restrict the building up of glycerophosphatides containing two long chain fatty acids in very close steric position.

Although the sphingomyelin molecule in its steric form has a resemblance to PC, it is obvious that the molecular position of one of the long chains, namely the sphingosine, is fixed, i.e., only one fatty acid has to be fitted into the structure. Furthermore, the fatty acid pool in EFA-deficient animals has a large content of monoenes and saturated acids which are the main fatty acids in normal sphingomyelins, whereas a high degree of unsaturated fatty acids is necessary in the glycerophosphatides.

The fatty acid composition of the NL and the PL of the generated and normal testes of rats will be reported later.

ACKNOWLEDGMENT

Miss Britta Offenberg gave technical assistance in these experiments.

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[Received November 18, 1968]

Differential Incorporation of Acetate and Glucose in Maturing Douglas Fir Seeds[1]

TE MAY CHING and **S. C. FANG**, Department of Farm Crops and Agricultural Chemistry, Oregon State University, Corvallis, Oregon 97331

ABSTRACT

In order to discern the synthetic pathways of lipids in coniferous seeds, decoated maturing Douglas fir seeds were incubated with 2-^{14}C-acetate and ul-^{14}C-glucose for 3 hr in phosphate buffer at pH 6.0. About 52% of incorporated acetate was found in lipids, but only 9% of the absorbed glucose was converted to lipids. Distribution of incorporated radioactivity in lipid classes was similar for both substrates, 45% in polar lipids, 22% in diglycerides, 15% in triglycerides, 7% in sterol esters, 4% in each of fatty acids and monoglycerides, and 3% in sterols. High specific activity was found in free fatty acids, diglycerides, monoglycerides and polar lipids indicating a rapid turnover of the intermediates for reserve triglycerides and structural polar lipids. Degradation analyses showed that 50% of incorporated acetate and glucose in lipids were in fatty acid moiety. Acetate contributed more in sterols and other unsaponifiables than in glycerol, and the reverse was true for glucose. All the data indicated that acetate is the direct precursor of fatty acids and sterols. General synthetic pathways prevail in fir seeds. Methods for complete analysis of chemical and radio-chemical composition were presented and results discussed.

INTRODUCTION

Mature Douglas fir seeds (*Psendotsuga menzienssii*, F.) contain 45% lipids (3,4). Their lipogenic pathways, however, have not been investigated yet even though incorporation of glucose to reserve lipids and structural components has been demonstrated in this seed (2).Radioactive glucose and acetate were used as tracers to discern the biosynthetic activities in maturing seeds.

MATERIALS AND METHODS

Maturing Douglas fir cones with seeds at the linear phase of dry weight accumulation (1), about 100 days after pollination, were collected. Seeds were dissected from the cones. Two replications of 1.25 g decoated seeds were incubated with shaking at 25 C for 3 hr in 10 ml of 0.1 M potassium phosphate (pH 6.0) containing 0.2 mmoles ul-^{14}C-glucose with an approximate radioactivity of 10 μc or 4 mmoles of 2-^{14}C-acetate of 8 μc.

Carbon dioxide was collected hourly in 50 ml 0.2 N NaOH. Two aliquots were counted in a Packard Tricarb liquid scintillation spectrometer after adding 10 ml counting solvent (5 ml toluene containing 20 mg 2,5-diphenyloxazole and 0.25 mg 2,2-paraphenylene bis 5-phenyloxazole and 5 ml ethyl cellusolve containing 0.25 g naphthalene). At the end of the incubation period, seeds were rinsed, surface dried and weighed. For further fractionating to various chemical components, the general procedure for Douglas fir seeds was followed (4). The seeds were first extracted by chloroform-methanol (2:1 v/v), and the extract was washed with water. The washed chloroform-methanol extracts contained total lipids from which two aliquots were taken and counted. The counting efficiency was calculated and the quenching effect corrected by adding 7-^{14}C-benzoic acid with known dpm to all samples as internal standard. Generally a counting efficiency of 50% was obtained.

An aliquot of the total lipids was separated into different classes on a silica gel G plate (250 μ in thickness) in petroleum ether-ether-acetic acid (70:30:1 v/v) (9). The plate was scanned by a Packard radiochromatogram scanner model 7201. The distribution of radioactivity in each lipid class was estimated from peak area of the radiogram. Quantitative thin layer chromatographic technique (11) was used to calculate the weight distribution in each class using cholesterol palmitate, triolein, oleic acid, diolein, β-sitosterol, monolein and phosphatidyl choline as standards. Specific radioactivity of each lipid class was calculated by dividing weight into the absolute radioactivity. For further tracing of the differential incorporation of the substrates, lipid classes were isolated from the thin layer chromatogram and saponified in 10% sodium hydroxide in 75% methanol. The unsaponifiables, fatty acids and water solubles were separated (3) and counted.

Further fractionation of the fat-free residue was conducted (4) for calculating total recovery and completeness of the tracer analysis.

[1]Technical paper 2346 Oregon Agricultural Experiment Station.

TABLE I

Distribution of Weight and Radioactivity, and Specific Incorporation in Various Fractions of Maturing Douglas Fir Seeds Incubated With 2-^{14}C-acetate and ul-^{14}C-Glucose

Substrate	Expt.	2-^{14}C-acetate			ul-^{14}C-glucose		
		Weight mg	Incorp.[a] %	Sp. act.[b]	Weight mg	Incorp.[a] %	Sp. act.[b]
CO_2	I	9.30	0.17	0.33	13.70	6.16	6.16
	II	10.60	1.08	1.81	15.60	6.82	5.72
Lipids	I	332.00	51.75	2.80	299.00	8.70	0.40
	II	306.00	51.70	3.12	265.00	8.61	0.42
Amino acids	I	9.74	16.32	33.00	10.08	6.94	9.56
	II	7.00	16.88	43.20	8.98	6.44	9.30
Anionic solubles	I	3.49	12.52	64.40	2.95	4.35	20.20
	II	3.54	9.12	49.40	2.41	4.42	24.00
Sugars	I	6.60	0.69	1.87	6.60	48.65	101.60
	II	7.60	0.64	1.45	5.80	53.45	121.30
Soluble proteins	I	1.00	0.26	4.68	1.52	0.07	0.72
	II	1.42	0.15	1.81	1.42	0.05	0.50
RNA	I	4.32	3.43	14.30	3.60	3.25	12.40
	II	3.85	3.77	16.70	3.71	3.78	13.40
DNA	I	0.32	0.15	7.92	0.39	0.28	10.10
	II	0.33	0.16	8.24	0.34	0.28	10.80
Insoluble proteins	I	114.00	1.29	0.21	126.00	1.51	0.16
	II	138.00	1.48	0.19	116.00	1.58	0.18
Starch	I	11.05	0.19	0.30	10.80	1.05	1.28
	II	10.44	0.32	0.54	11.92	1.09	1.24
Fibers	I	27.50	0.28	0.18	29.40	0.75	0.35
	II	29.50	0.32	0.19	28.60	0.87	0.38

[a]Incorporation, per cent of total recovery.

[b]Specific activity, mμc/mg.

RESULTS AND DISCUSSION

The reduction of radioactivity from media after 3 hr incubation was found to be 20%, of which 90% was recovered from different fractions.

Utilization of acetate was rapid and mainly for the synthesis of lipids (Table I). About 17% of the total radioactivity was found in the soluble cationic fraction composed chiefly of amino acids and amides, and only 11% radioactivity was observed in the anionic fraction containing mostly organic acids. Substrate ace-

TABLE II

Distribution of Radioactivity and Weight, and Specific Incorporation in Lipid Classes of Maturing Douglas Fir Seeds Incubated With 2-^{14}C-acetate and ul-^{14}C-Glucose for 3 hr at pH 6.0[a]

Substrate	2-^{14}C-Acetate			ul-^{14}C-Glucose		
	Weight mg	Radio-activity dpm x 10^{-3}	Sp. act. mμc/mg	Weight mg	Radio-activity dpm x 10^{-3}	Sp. act. mμc/mg
Sterol esters	3.8 ± 0.3	0.9 ± 0.2	7.22 ± 0.51	4.2 ± 0.4	5.0 ± 0.6	0.46 ± 0.08
Triglycerides	86.4 ± 1.7	16.9 ± 0.9	0.51 ± 0.06	86.3 ± 1.4	12.3 ± 1.1	0.06 ± 0.01
Free fatty acids	0.5 ± 0.1	3.4 ± 0.2	19.74 ± 1.66	0.2 ± 0.0	5.3 ± 0.3	11.18 ± 0.62
Diglycerides	1.6 ± 0.1	21.4 ± 1.2	40.65 ± 2.06	1.6 ± 0.2	22.8 ± 2.1	5.77 ± 0.67
Sterols	2.0 ± 0.1	2.0 ± 0.2	2.88 ± 0.29	1.7 ± 0.2	3.0 ± 0.1	0.73 ± 0.05
Monoglycerides and others[b]	0.4 ± 0.1	3.9 ± 0.3	24.35 ± 1.52	0.3 ± 0.0	3.8 ± 0.4	5.85 ± 0.62
Polar lipids[c]	5.0 ± 0.2	43.4 ± 2.7	24.45 ± 0.50	5.8 ± 0.4	47.8 ± 1.8	3.47 ± 0.18
Total	319 ± 3	2,040 ± 4	2.96 ± 0.11	282 ± 4	257 ± 2	0.41 ± 0.04

[a]The mean ± standard deviation of four replications.

[b]Mainly monoglycerides and esterified sterol glucosides.

[c]Phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl serine, phosphatidic acid, sterol glucosides, glycolipids and aminolipids.

TABLE III

Per Cent Distribution of Radioactivity in Various Moieties of Lipid Classes in Maturing Douglas Fir Seeds Incubated With 2-^{14}C-Acetate and ul-^{14}C-Glucose for 3 hr at pH 6.0[a]

Substrate moiety	2-^{14}C-Acetate				ul-^{14}C-Glucose			
	Total	Uns[c]	Soluble	FA[b]	Total	Uns[c]	Soluble	FA[b]
Sterol esters	9.0	5.6 ± 0.5	0.3 ± 0.0	3.1 ± 0.3	5.0	1.3 ± 0.2	0.5 ± 0.1	3.3 ± 0.4
Triglycerides	16.9	---	0.4 ± 0.0	16.5 ± 1.1	12.3	---	1.1 ± 0.1	11.2 ± 1.2
Fatty acids	3.4	---	---	3.4 ± 0.2	5.3	---	---	5.2 ± 0.3
Diglycerides	21.4	---	1.0 ± 0.1	20.4 ± 1.6	22.8	---	5.3 ± 0.6	17.5 ± 2.1
Sterols	2.0	2.0 ± 0.2	---	---	3.0	3.0 ± 0.1	---	---
Monoglycerides, etc.	3.9	2.2 ± 0.2	0.3 ± 0.0	1.4 ± 0.3	3.8	0.1 ± 0.0	1.6 ± 0.2	2.1 ± 0.1
Polar lipids, etc.	43.4	25.8 ± 3.1	7.4 ± 0.8	10.2 ± 1.0	47.8	10.5 ± 0.8	24.0 ± 1.7	13.3 ± 1.8
Total	100.0	35.6	9.4	55.0	100.0	14.9	32.5	52.6

[a]The mean ± standard deviation of 4 replications.

[b]FA, fatty acids.

[c]Uns, unsaponifiables, mainly sterols.

tate was evaporated as volatile solubles during fractionation, and the total volatile compounds constituted only 3% of the total radioactivity. A very small quantity of acetate was incorporated into sugars and soluble proteins, or channeled to the tricarboxylic acid cycle for respiration.

Glucose apparently was not a substrate for the synthesis of lipid reserves or cellular components since a major portion of radioactivity still remained in the neutral soluble fraction or sugars (Table I). Further characterization of this neutral fraction by paper chromatography showed that 75% of the radioactivity found in this fraction was in sucrose and the remaining 25% in glucose. Less than 9% of the radioactivity from glucose was incorporated into lipids, about 6% for respiration, 7% to soluble cationic and 4% to anionic compounds. Little incorporation of glucose into soluble proteins was found.

Both acetate and glucose contributed at a comparable level of about 11% and 14% respectively to the ethanol-insoluble, cellular structural components (Table I). The difference of the two radiochemicals was only in the starch and fibers, both of which could be directly synthesized from glucose. Approximately one third of the radioactivity in the insoluble fraction was found in RNA indicating rapid assimilation or turnover of this compound in the tissue.

Specific activity in tracer studies indicates the proportion of synthesized or turnover quantity to the total pool of a particular compound. All the soluble fractions had high specific activity indicating a rapid rate of metabolism in this material. Since the tissue used in this case is at the linear growth stage, synthesis probably exceeds turnover in reserve compounds and structural components. Biosynthesis of RNA and DNA was also very active, even though acetate and glucose are not the direct substrates for nucleic acids. Apparently developmental biosynthetic pathways are integrated according to the genetic control.

The differential incorporation of acetate and glucose into lipids had been observed in higher plants such as peanut cotyledons, runner bean leaves (7,10), and in lower fungus (6). The general trend was therefore comparable in three different taxonomic groups of plants.

The per cent distribution of incorporation of both acetate and glucose indicated that maturing Douglas fir seeds chiefly accumulated reserve glycerides and synthesized phospholipids and sterol esters for structural components of cellular organelles (Table II). Free fatty acids, diglycerides and monoglycerides are the intermediates of reserve and structural lipids (8) and their specific activities in fir seeds reflect such a role. Data in Table III further indicated that acetate was the substrate for the biosynthesis of fatty acids and sterol compounds, while glucose was partly oxidized via glycolysis to acetate which was then incorporated into fatty acids and sterols. Glycerol and other water soluble moieties of lipids are more directly related to glucose than to acetate, thus glucose contributes more than acetate.

Approximately 28% of the total incorporated acetate was in the fatty acid moieties of lipids compounds; this quantity is much higher than the incorporation by cereal seedlings and bean leaves (5,7). Tissue specificity is clearly indicated in these data since seedlings and leaves are not the site for assimilating glycerides.

ACKNOWLEDGMENTS

Mary E. McKillican, Food Research Institute, Canada Department of Agriculture, Ottawa, provided the sterol glucoside, esterified sterol glucoside, monogalactosyl glyceride and digalactosyl glyceride; Isabelle Schoolcraft, Betty Fallin and Kenneth Ma provided technical assistance. This work was supported in part by the National Science Foundation Grant GB-3187.

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[Received October 7, 1968]

Phase Relationships Involving the Deposition of Cholesterol From Triglyceride Solution

LEMUEL D. WRIGHT and **J. L. GAYLOR**, Graduate School of Nutrition and the Section of Biochemistry and Molecular Biology, Division of Biological Sciences, Cornell University, Ithaca, New York 14850

ABSTRACT

Adsorption isotherms describing the adsorption of cholesterol from solution in various oils by Permutit were investigated. Multiple layer adsorption onto Permutit and cholesterol crystallization from solutions that would have been subsaturated with respect to cholesterol in the absence of Permutit were observed. Association of cholesterol with Permutit, required for multiple layer adsorption, was observed even at low concentrations of cholesterol. Near infrared absorption spectra of solutions of cholesterol revealed marked aggregation of cholesterol in solutions of greater than about 0.2 M. Thus, association with Permutit apparently initiates crystallization and the similar aggregation of cholesterol in solution and in the solid phase apparently propagates the crystallization of cholesterol. Hydrogen bonding of cholesterol to the ester bond of triglycerides is unrelated to solubility of cholesterol in triglycerides examined.

INTRODUCTION

Previous studies from this laboratory have shown (1) that cholesterol is readily adsorbed from solution by Permutit. Adsorption isotherms describing the adsorption of cholesterol from solution in hexane by Permutit are of the familiar type $y = ax^n$, where $a > 1$ and $n < 1$. Adsorption isotherms describing the adsorption of cholesterol from solution in triglycerides by Permutit, however, are of the type $y = ax^n$ where $a < 1$ and $n > 1$. Such adsorption curves are typical of those encountered in multiple layer adsorption. In multiple layer adsorption one layer of adsorbate is first attracted with relatively low affinity to an adsorbing surface. The presence of this first adsorbed layer renders the surface more attractive to a second layer than to the first layer, and the presence of a second layer renders the surface more attractive to a third layer than to the second layer and so on in a vicious circle manner.

Multiple layer adsorption can occur from solutions that are not saturated with solute. The phenomenon described is well known physically but occurrence in systems modeled after physiological conditions may not have been pointed out previously. Thus, it is quite interesting to consider that under in vivo conditions, the adsorption of cholesterol from solutions onto a surface, such as, e.g., a component of the aorta may lead to the crystallization of cholesterol in deposits that would not otherwise have been encountered. In studies already described (1) cholesterol concentrations were investigated up to levels where $d^2y/dx^2 = 0$. Additional studies have now been completed involving equilibrium concentrations of cholesterol in triglyceride solutions up to and including saturation. The results obtained show that cholesterol crystallizes from solution at concentrations that would otherwise remain in solution in the absence of the solid Permitit. Apparently the chemical potential of dissolved cholesterol is elevated by solid Permutit.

Examination of the adsorption isotherms suggests that the process of multiple layer adsorption observed here is rather complex. Further, Parker and Bhaskar (2) demonstrated recently that cholesterol in solution is not a single, undissociated molecule. Indeed, concentration-dependent dimers and higher aggregates of cholesterol in carbon tetrachloride solution are observed. Further, when triglycerides are added to the solutions of cholesterol in carbon tetrachloride the cholesterol is hydrogen-bonded to the esters (2). Accordingly, cholesterol in solutions of solvents or natural oils is related to experimental conditions used in the studies of cholesterol adsorption isotherms (3-5). Conclusions regarding the forms of dissolved cholesterol, the contribution of aggregation to crystallization, and the multiple layer association of cholesterol on surfaces such as Permutit are described.

EXPERIMENTAL PROCEDURES

The initial procedures employed were essentially those utilized in previous solubility and adsorption studies in this laboratory (1,3-5). ^{14}C-Cholesterol of relatively low activity (215 cpm/mg; 280 dpm/mg) was weighed out in various amounts (25 to 240 mg) into screw-capped glass test tubes. Permutit (Eimer and Amend, according to Folin) was added (200 mg) to the dry cholesterol. It was determined that the Permutit contained no material Soxh-

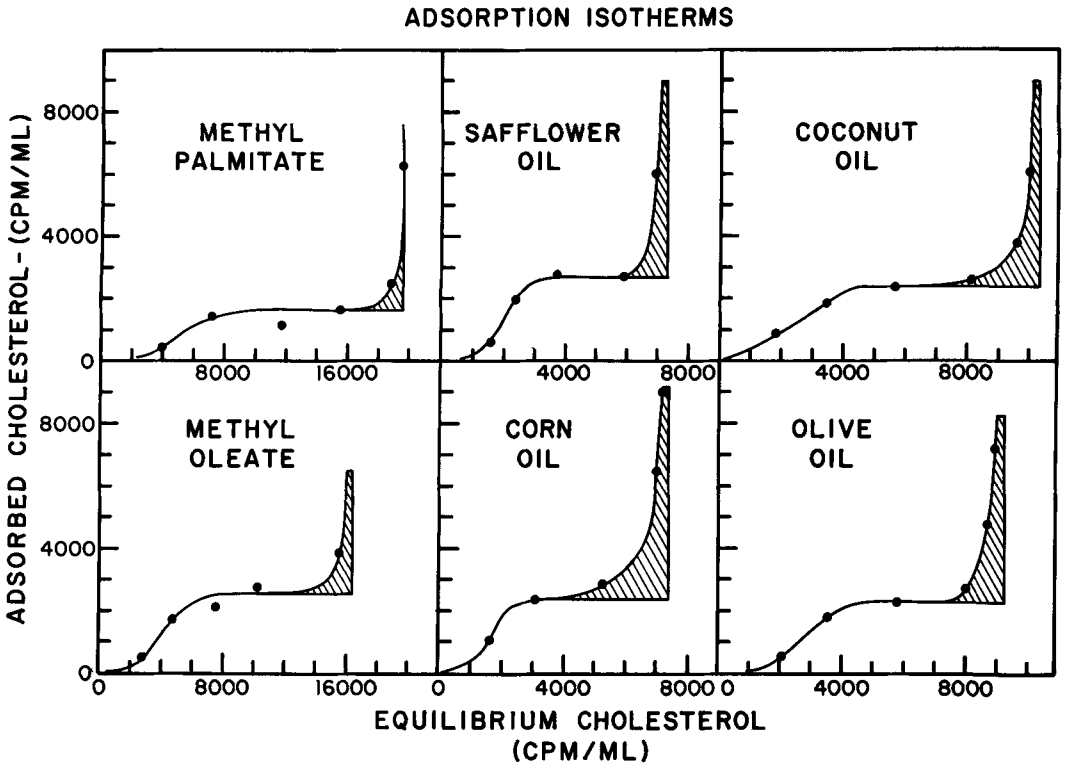

FIG. 1. Adsorption isotherms involving the adsorption by Permutit of cholesterol from solution in various esters and triglycerides.

let extractable by chloroform and only 0.05% of material extractable by leaching with hot water. Various triglycerides or methyl esters of fatty acids (2 ml) were added to the tubes. The tubes were then stoppered and placed in an apparatus that rotated the tubes end-over-end (60 rpm) at incubation temperature (37 C). Following rotation with incubation (18-24 hr), the tubes were centrifuged (10 min) in a clinical centrifuge maintained at incubation temperature. It may be calculated that in a typical experiment of 18 hr duration the tubes were exposed to 64,800 inversions. A separate experiment involving the solution of a saturating amount of cholesterol in safflower oil showed that solution is complete in 1-2 hr. Thus 18 hr of incubation involves the equilibration of cholesterol with triglyceride in excess of 900% of the time required to effect saturation. Aliquots of the supernatant solutions (ca. 1 ml) were transferred by disposable pipettes to tared counting vials. After weighing, scintillation solution [10 ml, 4 g PPO (2,5-diphenyloxazole)] and 30 mg POPOP [1,3-Dl-2(5-phenoxazoyl)-benzene per liter of toluene] were added to each vial, and the solutions were counted in a liquid scintillation spectrometer (Packard Tri-Carb). From the counts obtained and the radioactivity of the cholesterol used, the amount of cholesterol adsorbed at each equilibrium level of cholesterol in solution was readily calculated.

The triglycerides used were all commerical preparations intended for human consumption. The methyl esters of fatty acids were purchased from Nutritional Biochemicals Corp.

The presence or absence of cholesterol crystals dispersed in the triglycerides and not attached to the Permutit was determined with a microscope occular lens system (7.5x) used in the reverse direction. Cholesterol was characteristically crystalline, while the particles of Permutit were irregular in shape and buff in color.

The first overtone of the fundamental OH-stretching frequency of cholesterol was measured in the near infrared region with a Cary recording spectrophotometer (6). Crystalline cholesterol (mp 147-8°; $[\alpha]_D$ - 38.5°), purified via the dibromide, was dissolved in spectral grade chloroform (Mallinckrodt). Spectra were recorded from 1.380 to 1.500 μ. All measurements were made at 23 ± 2°.

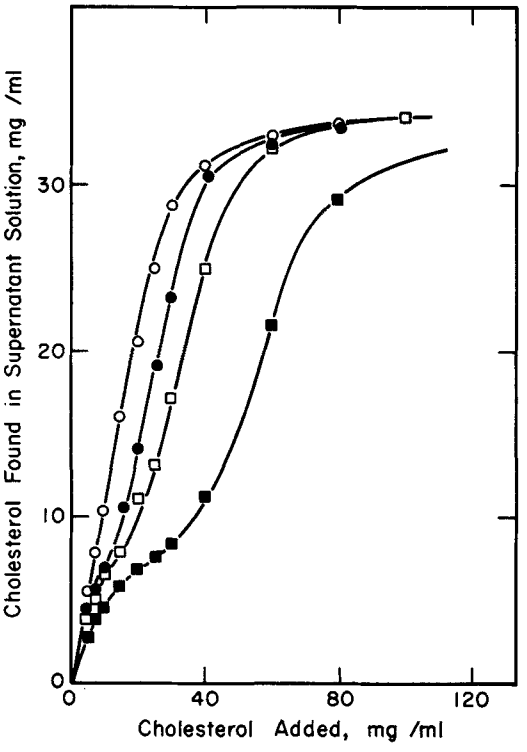

FIG. 2. The adsorption by various levels of Permutit of cholesterol dissolved in safflower oil. The tubes contained the following amounts of Permutit (per milliliter of oil): 0, ○—○; 50, ●—●; 100, □—□; and 250, ■—■.

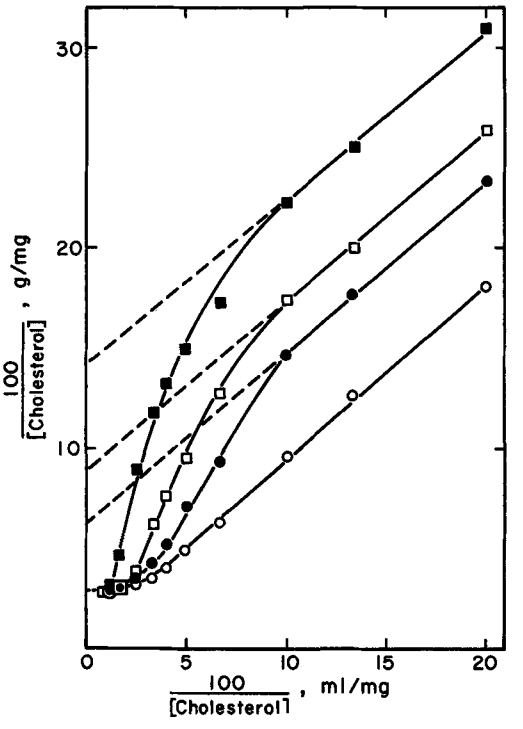

FIG. 3. Reciprocal plot of the effect of Permutit on the behavior of cholesterol in safflower oil (data of Fig. 2). The reciprocal of the amount of cholesterol remaining in the oil is plotted in units of milligram of cholesterol per gram of oil, and the reciprocal of the amount of cholesterol added is plotted in units of milligram of cholesterol per milliliter of oil. The key for the levels of Permutit is the same as that given in Figure 2.

RESULTS

Effect of Permutit on Solubility of Cholesterol in Oils

The results obtained are summarized in Figure 1. For the various triglycerides and methyl esters the amount of cholesterol adsorbed is plotted as a function of the corresponding equilibrium concentration of cholesterol. At equilibrium levels of cholesterol up to approximately one half of saturation, curves of the type $y = ax^n$, where $a < 1$ and $n > 1$ were obtained. For example, the curve fitting the data for low equilibrium levels of olive oil is described by $y = 0.0365 \times ^{1.5}$. At equilibrium levels above about one half of saturation, little or no additional cholesterol is adsorbed by the Permutit until saturation is approached. Here it is impossible, with the procedures employed, to distinguish between cholesterol adsorbed and cholesterol crystallized from solution. Crystalline cholesterol was readily observed, however, at equilibrium levels of cholesterol in the order of 80% or less of saturation in the absence of Permutit. With more refined procedures for the detection of cholesterol crystals, it is presumed that crystals would be observed at equilibrium levels well below those recorded. The shaded parts of the Figure indicate deviations from the expected adsorption-solubility curves. It is in these areas of concentration of dissolved cholesterol that crystals of cholesterol may be observed in suspension. Thus, due to the presence of Permutit and the associated phenomenon of adsorption, crystals of cholesterol are present in suspension at concentrations of dissolved cholesterol that would not have been seen had the Permutit and the adsorbed cholesterol not been present.

Figure 2 graphically shows the data obtained in more extended adsorption studies involving safflower oil as the triglyceride where levels of both added cholesterol and Permutit were varied. Plotted on the abscissa is the level of added cholesterol in mg/ml. Plotted on the ordinate is the level of cholesterol found in solution following equilibration with the indicated amounts of Permutit. This experiment

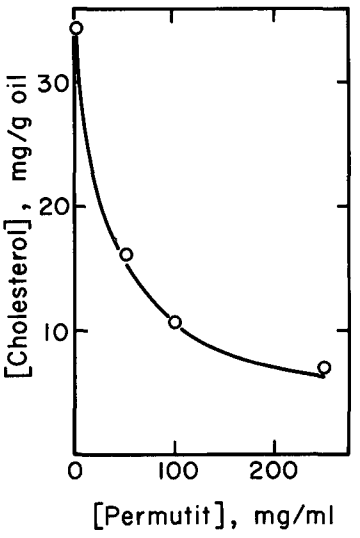

FIG. 4. Association of Permutit with cholesterol. Intercepts from Figure 3 are plotted (in mg/g oil) against Permutit added.

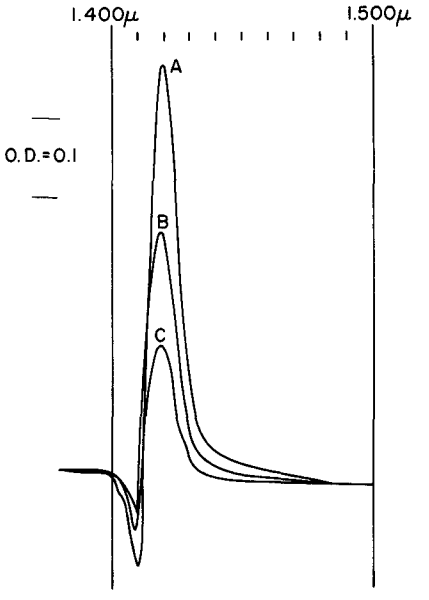

FIG. 5. Effect of cholesterol concentration on the λmax of the first overtone of the fundamental OH-stretching frequency. The concentrations of cholesterol in chloroform are: A, 0.518 M; B, 0.259 M; and C, 0.129 M.

involved the addition of safflower oil to the indicated amounts of dry cholesterol and Permutit in test tubes, followed by equilibration. Identical results were obtained over that part of the curves that could be duplicated where the cholesterol was dissolved prior to the addition of the Permutit. Thus the same equilibrium point is reached with respect to the adsorption of cholesterol on Permutit when approached from either side. It is apparent from the Figure that cholesterol is well adsorbed on Permutit from solution in safflower oil with the extent of adsorption roughly proportional to the amount of Permutit present. There are, however, certain anomalous parts of these curves which appear to be more readily explainable when the data are plotted in another way.

A reciprocal plot (Fig. 3) of the effect of Permutit on solubility of cholesterol in safflower oil was prepared from the data given in Figure 2. For each concentration of Permutit, three separate parts of each curve are visible. At very high concentrations of cholesterol (approximately 50 to 100 mg/ml), addition of each increment of cholesterol yields little or no change in the concentration of dissolved cholesterol. Extrapolation of this essentially horizontal line to the Y-intercept yields a calculated solubility of 32.2 mg of cholesterol dissolved per gram of safflower oil. A value of 32.8 mg/g oil (29.5 mg/ml oil) was reported by Kritchevsky and Tepper (7), and a value of 31.8 mg/g oil has been found in this laboratory by measuring solubility with saturating amounts of crystalline cholesterol. When the concentration of Permutit was high (250 mg added per milliliter of solution, the approach to the limit of 32.2 mg/g oil required more cholesterol, as expected.

A second part of the reciprocal plot lies between limits of 10 and 50 mg of cholesterol added per milliliter of oil. A complex process obviously occurs in this range of concentrations (approximately 0.026 M to 0.13 M). Indeed, within this range of concentrations of cholesterol in neutral solvents, aggregation of cholesterol in solution is observed (2). In addition, precipitation of crystalline cholesterol is clearly visible. Thus, in this range of concentrations, cholesterol aggregates both in solution and in the solid phase.

When the concentration of cholesterol is less than 10 mg/ml of oil (<0.026 M), linear portions of the reciprocal plots are observed. The linear portion was extrapolated to the Y-intercept and the concentrations of cholesterol in oil were calculated and plotted as a function of Permutit added (Fig. 4). Thus, at these low concentrations of cholesterol a very regular effect of Permutit addition on the equilibrium amount of cholesterol in solution is observed. Approximately 50% of the cholesterol partitions onto the solid when the amount of Permutit added is 50 mg/ml (Fig. 4).

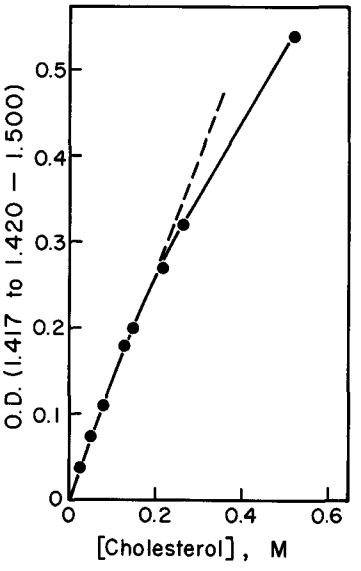

FIG. 6. Effect of cholesterol concentration on the absorbancy at 1.417 μ due to OH-stretching.

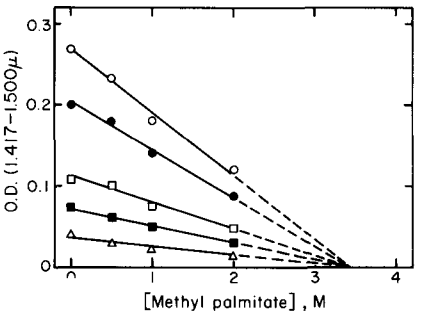

FIG. 7. Effect of methyl palmitate on the absorbancy of cholesterol at 1.417 μ. Methyl palmitate was added to solutions of cholesterol in chloroform: 0.208 M, ○–○; 0.149 M; ●–●; 0.083 M, □–□; 0.052 M, ■–■; and 0.025 M, Δ–Δ. The ΔO.D. between 1.417 μ and 1.500 μ is reported.

Effect of Triglycerides on Forms of Cholesterol in Solution

The three phases of cholesterol adsorption onto a solid cannot be interpreted unless the physical forms of cholesterol in solution are known. The forms of cholesterol in CCl_4 solutions and the effect of added glyceride were examined extensively by Parker and Bhaskar (2) with the use of infrared spectroscopy. Accordingly, we investigated the forms of cholesterol in solution by observing the sharp absorption peak of cholesterol in the near infrared region of the spectrum (6) because an instrument similar to the one used by Parker and Bhaskar was not readily available to us. Initially, conditions for the observation of this absorption peak and the quantification of absorbancy were examined.

When the concentration of cholesterol is rather low (<0.15 M) a λ_{max} of 1.417 μ is observed for cholesterol in chloroform solution (Fig. 5). When the concentration is greater than 0.2 M, the λ_{max} shifts to approximately 1.420 μ. The absorbancy at 1.417 to 1.420 μ is proportional to the concentration of cholesterol throughout the concentration range examined until the concentration exceeds 0.2 M (Fig. 6). The spectral changes observed here are related to cholesterol concentration in the same way as the spectral changes observed in the infrared region (2).

Next, the effect of addition of triglyceride and other esters to cholesterol solutions was investigated. First, the effect of addition of a simple ester, methyl palmitate, on the infrared absorption of cholesterol was examined. For all concentrations of cholesterol investigated, addition of methyl palmitate results in quenching of the absorbancy at 1.417 to 1.420 μ (Fig. 7). The amount of quenching is directly related to the amount of ester added. The concentration yielding complete quenching (3.5 M) is essentially the concentration of pure methyl palmitate.

Next, the effect of esters on quenching was extended to the natural oils used in the study of solubility (Ref. 1 and Fig. 1). The amount of quenching of absorbancy produced by methyl oleate is approximately equal to that observed for methyl palmitate (Table I). The extent of quenching produced by all of the natural oils is approximately equal. Furthermore, based on the number of ester bonds, the extent of quenching produced by the simple esters and the triglycerides is equal.

The solubilities of cholesterol in the various triglycerides are also reported in Table I for comparison. Obviously, solubility is rather variable, whereas the extent of quenching is uniform. Thus, solubility and quenching are not necessarily related.

DISCUSSION

The first overtone of the fundamental OH-stretching frequency (6) of the hydroxyl group of cholesterol may be examined easily with a Cary recording spectrophotometer. Changes in the observed λ_{max} and absorbancy agree well with the physical forms of cholesterol in solution reported by Parker and Bhaskar (2). When the concentration of cholesterol in chloroform solution exceeds about 0.2

TABLE I

Contents of Samples Showing Cholesterol Concentration and Percentage of Quenching[a]

Oil	Approximate concentration M	Per cent quenching	Solubility of cholesterol in oil[b] mg/ml
Methyl palmitate	1.85	52.5	---
Methyl oleate	1.68	47.7	---
Corn oil	0.62	49.2	35.9
Olive oil	0.62	52.5	37.2
Coconut oil	0.62	54.0	49.7
Safflower oil	0.62	50.0	29.5

[a]Each sample contained cholesterol (0.156 M) and oil (12.5 g), 0.62 M calculated as tripalmitin, in a total of 25 ml of solution in chloroform. The percentage of quenching

$$\frac{\Delta \text{ O.D. without} - \Delta \text{ O.D. with oil} \quad X \quad 100}{\Delta \text{ O.D. without oil}}$$

was calculated for each oil.

[b]From Kritchevsky and Tepper (7).

M a loss of absorbancy is observed (Fig. 6). This loss is ascribed to the formation of higher aggregates of cholesterol in solution (2). Further, when the concentration is approximately 0.2 M or more a shift of λ_{max} from 1.417 to 1.420 μ is observed (Fig. 5). When the concentration of cholesterol is between 0.014 and 0.2 M cholesterol is present as a dimer (2).

When esters are added to solutions of cholesterol in carbon tetrachloride, hydrogen bonding of cholesterol to the ester bond is observed (2). Similarly, the loss of absorbancy at 1.417 to 1.420 μ is ascribed to hydrogen bonding of the cholesterol to the ester bond (Fig. 7). An equilibrium constant:

$$K = \frac{[\text{Cholesterol} \cdot \text{methyl palmitate}]}{[\text{Cholesterol}]\ [\text{methyl palmitate}]}$$

$$K = 0.63\ \text{l}\cdot\text{mole}^{-1}$$

may be calculated for the 1:1 complex (2) from the data in Figure 7. The constant is approximately one third of that observed for a triglyceride (2.0 $\text{l}\cdot\text{mole}^{-1}$ was reported by Parker and Bhaskar). However, on the basis of concentration of ester bonds the molarity of triglyceride is approximately one third of that of methyl palmitate. Thus, the two equilibrium constants for hydrogen bonding of cholesterol to the ester agree well.

The self-association of cholesterol and the hydrogen bonding of cholesterol to the triglyceride solvents markedly complicate the adsorption isotherms observed for cholesterol (Fig. 1). However, the concentration-dependent changes in the forms of cholesterol in solution correspond well to the three regions of the adsorption isotherms observed (Fig. 3). Further, these observations permit partial explanation of three observations reported here and earlier (3-5). First, multiple layer adsorption is observed. Adsorption isotherms of the type, $y = ax^n$ where $a < 1$ and $n > 1$, require association of cholesterol with Permutit. Even at low concentrations of cholesterol, association of cholesterol with Permutit is observed (Fig. 3 and 4). Secondly, when a clathrate-forming agent, such as pimelic acid, is added to solutions of cholesterol in various oils, the limiting amount of cholesterol removed from solution by clathrate formation is 50% (3). Higher aggregates of cholesterol in solution are formed when the concentration exceeds about 0.06 M (2) and marked aggregation, in solution, is observed with concentrations greater than 0.2 M (Fig. 6). Aggregation in solution thus accompanies aggregation in the solid phase. Because the energies involved in each process of aggregation may be similar, it is not surprising that added cholesterol partitions about equally into the solid and liquid phases to yield the limit of 50%. This phenomenon apparently is a property of cholesterol because it is observed for all oils, although the solubility of cholesterol in the oils varies widely (Ref. 4 and Table I). Third, cholesterol crystallizes from solutions that in the absence of Permutit would be subsaturated with respect to cholesterol because association with Permutit, even at low concentrations of cholesterol (Fig. 3 and 4), apparently serves to nucleate the process of crystallization. Once again, following nucleation, aggregation in the solid phase may be about equal energetically to aggregation in the liquid phase.

Finally, solubility of cholesterol in various

oils is related to factors other than hydrogen bonding of the hydroxyl group of the sterol with the esters because quenching of absorbancy and solubility are not correlated (Table I). Apparently solubility is directly related to the interaction of cholesterol with the side chains of the fatty acids.

There may be an analogy between precipitation of cholesterol under the in vitro conditions described here and the occurrence of cholesterol deposits in vivo. In the presence of cholesterol adsorbed on a physiological surface exposed to serum, additional cholesterol could be deposited at only intermediate serum lipid levels of cholesterol.

ACKNOWLEDGMENTS

This work was supported in part by research grant G-332, from the National Science Foundation, by research grants AI-01891, AM-10767 and HE-04138 and Career Award K6-AM-16,612, from the National Institutes of Health, and in part by funds made available through the State University of New York.

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[Received October 2, 1968]

The Effects of Catecholamines on the Distribution of ^{14}C-Cholesterol in Dogs

JOSEPH H. GANS and **MARILYN R. CATER**, Department of Pharmacology, University of Vermont College of Medicine, Burlington, Vermont 05401

ABSTRACT

Fifteen adult male pure bred Beagle dogs were given 4-^{14}C-cholesterol and a subsequent 18 day period was permitted to elapse for equilibration of the ^{14}C-cholesterol between blood and tissue compartments. Peanut oil, epinephrine in oil or norepinephrine in oil was administered by daily subcutaneous injections over a period of 63 to 81 days. Epinephrine but not norepinephrine resulted in sustained increases in plasma cholesterol concentrations. The half-life of ^{14}C-cholesterol in plasma was 19 days in control dogs, 18 days in catecholamine treated dogs. The slope of the decline in the specific radioactivity of plasma cholesterol did not change during epinephrine administration, suggesting an influx into plasma of endogenous cholesterol. Specific radioactivity of thoracic aorta cholesterol was fourfold greater than that of plasma cholesterol in control dogs at the end of the experiment and 1.3-fold greater than the specific radioactivity of cholesterol in the lower aortic segments. Treatment with epinephrine decreased the specific radioactivity cholesterol in all segments of the aorta and resulted in cholesterol specific radioactivity from thoracic aorta which was less than that of cholesterol from the lower aortic segments. Norepinephrine treatment resulted in decreases in the specific radioactivity of cholesterol from the thoracic and abdominal aortic segments but the changes were smaller than those observed in epinephrine treated dogs.

INTRODUCTION

Daily administration of epinephrine in oil to dogs results in sustained increases in plasma cholesterol concentrations (1). This response to epinephrine administration is not the result of increased hepatic cholesterol synthesis (2). Preliminary studies in dogs given 4-^{14}C-cholesterol demonstrated that the slope of the specific radioactivity time curves for plasma ^{14}C-cholesterol did not change during epinephrine administration despite significant increases in plasma cholesterol concentrations (3). When epinephrine was administered to dogs over periods of 32 to 48 days, the specific radioactivity of plasma cholesterol at the end of the experiment was the same in both control and epinephrine treated dogs (4). These results suggest that epinephrine-induced increases in plasma cholesterol concentrations may result from the influx of endogenous or exchangeable cholesterol into the plasma-liver cholesterol pool.

Long term epinephrine administration to dogs does not alter the distribution of ^{14}C-cholesterol between plasma and most tissues, an apparent exception being the aorta (4,5). Several weeks after the administration of 4-^{14}C-cholesterol, the specific radioactivity of cholesterol in the thoracic aorta is approximately fourfold greater than plasma cholesterol and 1.6-fold greater than the specific radioactivity of cholesterol from the lower aortic segments (4). Long term epinephrine treatment tends to equalize the specific activity of cholesterol throughout the aorta (4).

The experiments summarized here were performed in order to study, over longer time periods, the effects of two catecholamines, epinephrine and norepinephrine, on the distribution of 4-^{14}C-cholesterol in dogs. Specific objectives of these experiments were an attempt to define the source of cholesterol which provides for the increased plasma concentrations of cholesterol in epinephrine treated dogs, to confirm the effects of epinephrine and to determine any effects of norepinephrine as well, on the distribution of ^{14}C-cholesterol in the aorta of dogs.

EXPERIMENTAL PROCEDURES

Fifteen adult male pure bred Beagle dogs were the subjects of this study. Each dog was immunized against distemper and infectious hepatitis and was maintained in the research animal facilities for two weeks prior to being included in the experiment. The dogs were permitted access ad lib. to water and to a dry dog food preparation (Purina Dog Chow, Ralston Purina Co., St. Louis, Mo.). Our analyses indicated that total lipid concentration of this diet is 10.2% and total cholesterol concentration is .075% on a dry weight basis.

TABLE 1

Effect of Catecholamines on Plasma Cholesterol Concentrations in Dogs

Group	Plasma cholesterol (mg/100 ml)							
	Start[a]	3 days	p(t)[b]	Start[c]	3 days	p(t)[b]	Final	p(t)[b]
Control (N=6)	130 ± 9.4	135 ± 15.5		121 ± 10.3	128 ± 9.6		133 ± 7.4	
Epinephrine (N=5)	147 ± 8.3	248 ± 35.6	<.02	145 ± 5.2	186 ± 13.2	< .01	185 ± 8.9	< .01
Norepinephrine (N=4)	128 ± 14.6	162 ± 20.0	<.20	126 ± 9.3	140 ± 12.7	<0.40	146 ± 14.1	<0.50

[a]Values at start of experiment, and after three days of treatment.

[b]Significance of difference between the catecholamine treated group and the control group. All data in this and subsequent Tables are expressed as the average ± 1 standard error of the mean.

[c]Plasma cholesterol concentrations taken during the mid-point of the experiment on a Monday morning immediately following a two day respite from treatment, and after three days of treatment.

Epinephrine in oil (Adrenalin in oil) was obtained from Parke-Davis Co., Detroit, Mich., and l-norepinephrine bitartrate was obtained from the Winthrop Laboratories, Inc., New York, N.Y. The norepinephrine was suspended in peanut oil by homogenization in a glass tube using a Teflon pestle.

4-^{14}C-cholesterol (specific radioactivity 58.0 mc/mmole, obtained from New England Nuclear Corp., Boston, Mass.) was administered intravenously to each of 15 dogs in a dose of 5 μc ^{14}C/kg body weight according to a method previously described (4). Fourteen, 16 and 18 days after the administration of the labeled cholesterol, blood samples were drawn for the determination of plasma cholesterol concentrations and of plasma cholesterol specific radioactivity.

Treatment was begun on the 18th day following the administration of the ^{14}C-labeled cholesterol. The doses of vehicle and drugs used were: 0.5 ml peanut oil per kilogram per day for each of six dogs in the control group, epinephrine in oil 0.8 to 1.0 mg/kg per day to each of five dogs and norepinephrine 0.8 to 1.0 mg equivalent of the free base per kilogram per day to each of four dogs. The vehicle or drugs were given subcutaneously for 12 days beginning on a Monday and the dogs were permitted a two day (Saturday and Sunday) respite from treatment. Through an ensuing period of seven to eight weeks the vehicle and drugs were administered five days per week on Monday through Friday inclusive. A final period comprising 10 to 12 days of consecutive treatment were completed prior to terminating an experiment. The total experimental period for 13 of the dogs, from the day in which 4-^{14}C-cholesterol was given to termination, ranged from 91-100 days, and treatment with the vehicle or the catecholamines ranged from 73 to 82 days. Limitations in time forced the termination of two experiments (one control and one norepinephrine treated dog) after 81 days which represented a treatment period of 63 days.

Blood samples for the determination of plasma cholesterol concentrations and specific radioactivity were taken at weekly intervals throughout the experiment and on the day in which each experiment was terminated. Additional blood samples were taken twice during the course of the experiment, on the third day of a treatment period which followed a two day respite. The specific radioactivity of plasma cholesterol was determined from all samples, and the data were used to determine the half-life of plasma ^{14}C-cholesterol. At randomly selected days during a course of treatment, blood samples were taken prior to and at 2, 4, and 7 hr after the injection of the vehicle or the catecholamine for the determination of plasma glucose concentrations.

Each experiment was terminated by anesthetizing the dog with pentobarbital sodium, 30 mg/kg, and by subsequent exsanguination. Sections of the following tissues were taken for the determination of cholesterol concentrations and specific radioactivity: heart, lungs, aorta (separated into thoracic, abdominal and terminal, kidney, adrenal glands, pancreas, liver, skin, skeletal muscle, renal artery and carotid artery.

Cholesterol concentrations in plasma and in tissues were determined according to methods previously described (4). Cholesterol for radioassay was isolated as the digitonide (6) and dissolved in 1 ml of methanol. A 0.2 ml aliquot of the methanol solution of cholesterol digitionide was taken for colorimetric estimation of cholesterol concentration; the balance was added to 10 ml of a liquid scintillation mixture (7) and radioactivity was assayed in a liquid scintillation spectrometer.

TABLE II

Specific Radioactivity of Blood Cholesterol and Half-Life of Total Plasma Cholesterol in Dogs Treated With Peanut Oil (Control), With Epinephrine in Oil and With Norepinephrine in Oil

	Specific radioactivity (cpm/mg)				
		End[b]			
		Plasma			
Group	Start[a]	Total	Free	Erythrocyte	Half-life[c] in days
Control	2,663 ± 235	158 ± 19.3	149 ± 20.0	162 ± 17.0	19 ± 1.3
Epinephrine	2,659 ± 162	125 ± 14.0	117 ± 15.3	116 ± 14.9	18 ± 1.1
Norepinephrine	2,631 ± 245	135 ± 42.4	117 ± 32.2	117 ± 40.7	18 ± 0.9

[a]Specific radioactivity of total plasma cholesterol taken 18 days after the administration of 4-^{14}C-cholesterol, prior to the start of treatment.

[b]Specific radioactivity of plasma and erythrocyte cholesterol taken on the day in which the experiment was terminated.

[c]Total plasma cholesterol.

Total lipid was extracted from adrenal tissue, kidney, liver and thoracic aorta according to the method of Folch et al. (8), and the total lipid extracts were separated into their major classes by preparative layer chromatography on glass plates coated with Silica Gel G (9,10).

The specific radioactivity of free and esterified tissue cholesterol from six samples of adrenal gland and two samples of liver was determined by eluting these fractions from silica gel following preparative layer chromatography. The specific radioactivity of each fraction did not differ markedly and was not different than the specific radioactivity of the total tissue cholesterol. Additionally, the specific radioactivity of free cholesterol from three samples of thoracic aorta, two samples of kidney cortex, one sample of kidney medulla and one sample of liver was determined following precipitation of free cholesterol with digitonin from the total lipid extract. These specific radioactivities of free cholesterol did not differ from the specific radioactivity of total cholesterol from each sample. The data which will be outlined and discussed, therefore, refers to the specific radioactivity of total tissue cholesterol.

Glucose concentrations in plasma were determined with glucose oxidase (Glucostat, Worthington Biochemical Co., Freehold, N.J.).

Criteria for the absorption of the catecholamines were (a) increased plasma glucose concentrations 4 and 7 hr after injection of the drig; (b) palpitation of the heart against the thoracic wall which was both visible and palpable within 2 hr and persisting for at least 7 hr after giving the catecholamine; and (c) periods of prominent bradycardia particularly in norepinephrine treated dogs.

RESULTS

Epinephrine administration to dogs was accompanied by increased total plasma cholesterol concentrations at the start of the experiment as well as at later time periods (Table I). Norepinephrine did not produce significant increases in plasma cholesterol concentration (Table I). Both catecholamine preparations increased plasma glucose concentrations.

The decline of the specific radioactivity of total plasma cholesterol was not changed by epinephrine administration, but total plasma radioactivity increased during epinephrine administration. Two experiments, typical of the patterns observed in control and epinephrine treated dogs are shown in Figure 1. During a two day respite from treatment, both plasma cholesterol concentrations and total plasma radioactivity decreased rapidly to control levels (Fig. 1). Plasma cholesterol specific radioactivity was the same in catecholamine treated dogs as in control dogs at the start of the experiment and did not differ significantly at the termination (Table II). The specific radioactivities of free plasma cholesterol and erythrocyte cholesterol in control and catecholamine treated dogs were not significantly different (Table II). The half-life of plasma cholesterol was 19 days in the control dogs and 18 days in the catecholamine treated dogs (Table II).

Specific radioactivity of cholesterol in the thoracic aorta was fourfold greater than the specific radioactivity of plasma cholesterol in the control dogs maintained on the basal dry

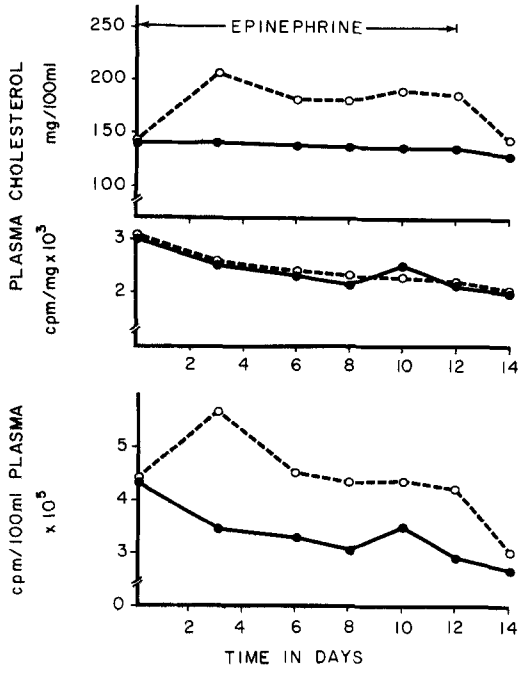

FIG. 1. Total plasma cholesterol concentrations and specific radioactivity and total plasma radioactivity in a dog given peanut oil (control), solid circles, and in a dog given epinephrine in oil, open circles. The responses of these dogs were typical of the group treated with peanut oil (control, N = 6) and of the group treated with epinephrine (N = 5).

food diet (Fig. 2). Thoracic aorta cholesterol specific radioactivity in control dogs also was greater than that of cholesterol from the lower segments of the aorta (Fig. 2, Table III).

The specific radioactivity of cholesterol from the three segments of the aorta was significantly decreased in epinephrine treated dogs (Fig. 2). The specific radioactivity of cholesterol in the thoracic aorta from epinephrine treated dogs was less than that from the lower segments, changes from the control relationships which were highly significant (Table III, Fig. 2). Norepinephrine produced smaller decreases in the specific radioactivity of cholesterol from the aorta, changes which were significant for the thoracic and abdominal segments (Table III).

Cholesterol specific radioactivities of the renal and carotid arteries were considerably greater than the specific radioactivity of plasma cholesterol (Fig. 2). The specific radioactivities of cholesterol of other tissues were within the range of plasma cholesterol specific activity and no significant changes were produced by either of the catecholamines (Fig. 2).

There were no differences in tissue cholesterol concentrations between the three groups except for kidney medulla in which the cholesterol level of the norepinephrine group was 28% higher than that of the controls [$p(t) < .01$].

DISCUSSION

The discussion of the results rests upon the assumption that ^{14}C-cholesterol in blood and in tissues, after a suitable period to permit equilibration, is representative of the exchangeable cholesterol in these compartments. Exchangeable cholesterol is composed of: (a) dietary or exogenous cholesterol which may be absorbed, (b) cholesterol which is secreted into the intestinal lumen in bile, (c) cholesterol excreted into the intestinal lumen by the intes-

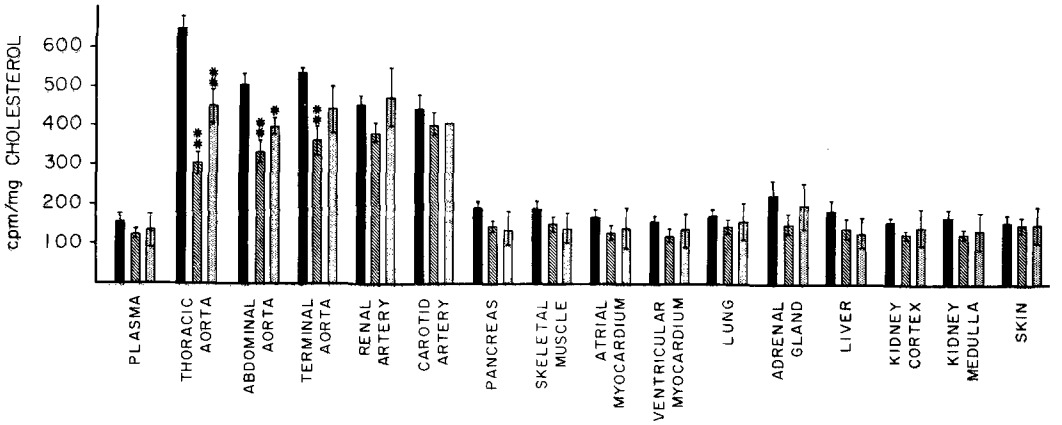

FIG. 2. Specific radioactivity of plasma and tissue cholesterol from dogs treated with peanut oil (control), solid black bars; epinephrine in oil, cross hatched bars; and norepinephrine in oil, dotted bars. **, Significance of difference from control: $p(t) < .01$; *, Significance of difference from control: $p(t) < .05$.

TABLE III

Effect of Catecholamines on the Distribution of ^{14}C-Cholesterol in the Aortas of Dogs

Group	SRA Th Ao[a] / SRA Ab Ao	p(t)[b]	SRA Th Ao[c] / SRA Ter Ao	p(t)[b]
Control	1.31 ± .09		1.22 ± .06	
Epinephrine	0.92 ± .03	<.01	0.85 ± .04	<.001
Norepinephrine	1.15 ± .14	<.10	1.08 ± .14	<0.50

[a]Specific radioactivity of the thoracic aorta cholesterol: specific radioactivity of the abdominal aorta cholesterol.

[b]Significance of difference from the control values.

[c]Specific radioactivity of the thoracic aorta cholesterol: specific radioactivity of the terminal aorta cholesterol.

tinal mucosa some of which may be reabsorbed, and (d) endogenous cholesterol synthesized primarily by the liver and perhaps to a very small extent by the intestinal mucosa (18). The cholesterol content of the diet was very low, although not negligible, and it is probable, therefore, that ^{14}C-cholesterol dynamics in these dogs is a reflection of the metabolism of endogenous cholesterol.

The possibility that diet, drugs or hormones may influence the distribution of cholesterol has been suggested by several reports which were concerned with the mode of action of plasma cholesterol lowering agents. Bieberdorf and Wilson (11) noted that the decreases in plasma cholesterol concentrations in rabbits given diets high in unsaturated fats could best be explained by a redistribution of cholesterol between plasma and tissues. Similar conclusions may be drawn from the clinical studies of Miller et al. (12) and Wollenweber et al. (13), who reported on the hypocholesterolemic effects of nicotinic acid in human subjects. Mietinnen (14) has shown that thyroxine reduces plasma cholesterol concentrations in hypothyroid patients by augmenting the fecal excretion of neutral sterol and possibly by altering the distribution of cholesterol as well.

Increased plasma cholesterol concentrations during epinephrine administration to dogs results from the influx of endogenous or more accurately, exchangeable cholesterol into the plasma-liver pool. This interpretation seems warranted since the slope of the specific radioactivity time curve for plasma ^{14}C-cholesterol and the half-life of plasma ^{14}C-cholesterol were the same in control and epinephrine treated dogs despite epinephrine-induced increases in plasma cholesterol concentrations.

These experiments confirm again that epinephrine does not stimulate hepatic cholesterol synthesis (2), since such an event would have increased the decline in the specific radioactivity of plasma cholesterol. It is unlikely that increased intestinal absorption is important in this response to epinephrine for in experiments in which 4-5 g of cholesterol were fed daily, epinephrine administration did not produce any evidence of an influx of unlabeled cholesterol (3). Aorta cholesterol also can not be the source of mobilized cholesterol; the average weight of the aorta is 4.0 g/10 kg body weight and total cholesterol in the aorta therefore would be between 8 and 10 mg. If all of the aorta cholesterol were mobilized into plasma this would account for only a small portion of the 120-150 mg of cholesterol which comprise the increase in plasma induced by epinephrine. A decrease in cholesterol degradation to bile acids seems not to be the route by which increased cholesterol is made available to the liver plasma pool for epinephrine may actually enhance the turnover and production of bile acids in dogs (15). Some cholesterol compartment in peripheral tissue which is readily available for redistribution must be the source of this increased plasma cholesterol in the epinephrine-treated dog.

These experiments confirm and extend the results of a previous study (4) of ^{14}C-cholesterol distribution in the dog. A longer period of both treatment and total duration of the experiments demonstrates clearly that cholesterol in the aorta of dogs exchanges very slowly with plasma cholesterol and that this relationship and the distribution of ^{14}C-cholesterol in the aorta are altered by long term daily subcutaneous administration of either norepinephrine or epinephrine in oil, the effects of epinephrine being more pronounced. The physiological significance of these effects of the catecholamines on ^{14}C-cholesterol distribution remains undefined. Possibly the functional and metabolic responses to the catecholamines,

particularly to epinephrine, may permit an accelerated interchange between cholesterol in plasma and cholesterol in the aorta of dogs.

We cannot explain our failure to observe significant increases in plasma cholesterol concentrations in norepinephrine-treated dogs since Barrett (16) and Barrett and Thorp (17) have reported that daily injections of l-norepinephrine bitartrate suspended in oil produces significant increases in plasma cholesterol concentrations of dogs. The effects of norepinephrine on plasma glucose concentrations as well as on the duration of bradycardia and cardiac palpitation indicate that norepinephrine was adequately absorbed. Our results indicate a differential effect of the catecholamines on cholesterol distribution in the dog with epinephrine exerting a significantly more impressive response. Similarly, epinephrine produces a more prominent effect on bile acid metabolism in dogs than does norepinephrine (15).

ACKNOWLEDGMENTS

This study was supported by U.S. Public Health Service Grant HE 11681 from the National Institutes of Health. A preliminary report was presented at the 1969 Meeting of the Federation of American Societies for Experimental Biology. Thanks to G. Melkonian for care of the dogs and for performing some of the cholesterol assays.

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[Received February 21, 1969]

Desaturation of Palmitate and Stearate by Cell-Free Fractions From Soybean Cotyledons[1]

JAMES A. INKPEN[2] and FORREST W. QUACKENBUSH,
Department of Biochemistry, Purdue University, Lafayette, Indiana 47907

ABSTRACT

Homogenates of cotyledons from immature soybean seeds were fractionated by centrifugation. Cell-free preparations actively desaturated 1-^{14}C-palmitate and 1-^{14}C-stearate to produce 9,10-unsaturated acids. The 9-desaturase activity was present mainly in supernatant fractions (22,000 and 105,000 x *g*). These fractions also desaturated oleate to linoleate and elongated C_{14}, C_{16} and C_{18} acids. In view of this versatility of desaturase systems in the soybean, including the 9-desaturase(s) for C_{14}, C_{16} and C_{18} saturated acids, there would not seem to be further need to consider a separate plant pathway for biosynthesis of unsaturated fatty acids.

INTRODUCTION

Although desaturation of the common saturated fatty acids, palmitic and stearic, by subcellular fractions from tissues of animals and microorganisms is well documented, similar efforts to study desaturation with comparable fractions or tissues from plant sources often have met with little success (1-3). Alternative pathways for the formation of oleic acid in plants have been sought (4,5). The active enzyme system in animal tissues is found in the microsomes (6); in yeast it is in the particulate fraction (7); and in Euglena it is found in the soluble fraction (8).

In earlier work this laboratory demonstrated that homogenates from soybean cotyledons desaturated uniformly-labeled palmitate and stearate (9). Subsequently, a system capable of desaturating stearate but not palmitate in spinach chloroplasts was reported (8). The present paper describes an extension of experiments (10) with cell-free preparations from soybean cotyledons which perform 9,10-desaturation reactions with 1-^{14}C-palmitate and 1-^{14}C-stearate.

EXPERIMENTAL PROCEDURE

Preparation of Cell-Free Fractions

Cotyledons (variety Harosoy), 35-40 days after flowering (11), were homogenized (Virtis, 2 min, 0 C) with a 5 fold quantity of 0.25 M sucrose-0.01 M potassium phosphate, pH 6.0. The homogenate was filtered through cheesecloth and the filtrate was centrifuged to remove cell debris. A floating lipid layer was removed by decanting the supernatant fraction onto glass wool. The resulting cell-free extract was adjusted to 6 mM with glutathione and 0.67 μM with disodium EDTA and then centrifuged at 16,000 or 22,000 x *g* for 1 hr. The sediment was resuspended in the original volume of cold buffered sucrose and designated the mitochondrial fraction. A lipid layer was again removed from the supernatant fraction before centrifuging at 105,000 x *g* for 90 min. The pellet (105,000 x *g*) was resuspended in the original volume of cold buffered sucrose and designated the microsomal fraction. The supernatant fractions obtained at 22,000 and 105,000 x *g* were used directly.

Substrates

The 1-^{14}C fatty acids (lauric, 1.88 mC per mmole; myristic, 13.0 mC per mmole; palmitic, 13.5 mC per mmole; stearic, 6.5 mC per mmole; and oleic, 5.3 mC per mmole) all were purchased from Calbiochem, Inc. and solubilized in a 1% solution of bovine serum albumin (defatted, Calbiochem, Inc.) as follows. An ether solution of the fatty acid (0.01 to 0.05 μmole) was evaporated in a 10 ml volumetric flask and 0.25 ml ethanol added to redissolve. After addition of 4 to 5 ml of 1% bovine serum albumin and shaking of the contents for 1/2 hr at 37 C, the flask was filled to volume with 1% BSA. Examination of the 1-^{14}C acids by thin layer chromatography ($AgNO_3$) showed purities of the saturated acids to be not less than 98%, with no measurable unsaturated contaminant. The 1-^{14}C-oleate contained 1.5% of polyunsaturated contaminant.

Assay for Fatty Acid Conversion

To a 25 ml Erlenmeyer flask was added 5 ml of the soybean fraction and the substrate dissolved in 0.1 or 0.2 ml of a solution containing

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[1]Published as Journal Paper No. 3197, AES, Purdue University, Lafayette, Indiana 47907.

[2]Present address: Parke, Davis and Company Research Laboratories, Ann Arbor, Michigan 48106.

TABLE I

Products Obtained From Incubation of Saturated Fatty Acids With a Soluble Fraction (105,000 x *g* Supernatant) From Cotyledons[a]

Substrate acid	Incubation time min	Radioactivity recovered in isolated fatty acid fractions, %			Radioactivity from monoene fragments as dicarboxylic acids, %
		Saturated	Polyene	Monoene	
1-^{14}C-Stearic	30	96.2	0.9	2.9	92.0
	60	91.0	2.7	6.2	92.4
	120	84.5	5.2	10.2	92.7
1-^{14}C-Palmitic	30	94.4	1.9	3.6	89.5
	60	90.1	3.1	6.9	90.5
	120	87.5	4.1	8.4	89.7
1-^{14}C-Myristic	30	90.8	2.2	6.8	92.7
	60	82.2	3.6	14.1	92.9
	120	80.4	3.7	15.9	91.9
1-^{14}C-Lauric	30	94.9	1.7	3.4	92.5
	60	90.9	3.2	5.9	86.0
	120	88.1	3.8	8.1	79.3

[a]The labeled fatty acids (0.01 to 0.05 μmole containing 0.3 to 0.5 μC of radioactivity) complexed with bovine serum albumin was added to a solution of five cofactors (ATP, 10 μmoles; coenzyme A, 0.27 μmole; NADPH, 0.2 μmole; $MgCl_2$, 2.5 μmoles; $MnSO_4$, 2.5 μmoles) in 5 ml of supernatant fraction containing the equivalent of 1 g of cotyledons. Total volume 5.1 ml per flask. Flasks were shaken at 28 C, methanolic KOH and carrier acids were added and the fatty acid esters separated by TLC. The monoenoic fraction was oxidized with periodate-permanganate and the acidic fragments were separated by TLC. Polyene and monoene data were corrected for radioactivity in comparable boiled control samples.

ATP, 10 μmoles; coenzyme A, 0.27 μmole; NADPH, 0.2 μmole; $MgCl_2$, 2.5 μmoles, and $MnSO_4$, 2.5 μmoles. The flasks were sealed with serum caps and shaken at 27-28 C (Dubnoff shaking incubator). Carbon dioxide was collected on termination of some of the reactions by injecting 0.5 ml of Hyamine hydroxide into a shell vial (35 x 10 mm ID) originally enclosed in the flask, then acidifying by injecting 0.5 ml of 6 N sulfuric acid into the incubation medium and shaking 1 hr longer. The shell vial was then removed, the outside surface rinsed and dried, and the vial and contents were inverted into a Wheaton flask of scintillation fluid.

TABLE II

Recovery of Label in Products From Schmidt Decarboxylation of 16:1 Fraction Derived From Palmitic 1-^{14}C Acid[a]

Replicate	Total radioactivity, cpm		Distribution of radioactivity, %	
	In sample	Recovered in $^{14}CO_2$	CO_2	Amine
1	4,280	3,211	84.5	2.9
2	3,640	2,600	81.4	2.3
3	4,015	3,442	94.2	5.7

[a]Palmitoleic acid obtained from the action of the 22,000 X *g* supernatant fraction upon 1-^{14}C-palmitic acid was isolated by TLC ($AgNO_3$) and GLC and subjected to Schmidt decarboxylation. The resulting CO_2 was collected and the fatty amine was extracted with ether after adjusting to pH 11-12.

In other cases, reactions were terminated on addition of 15 ml of 20% KOH in methanol and at this point about 5 mg of a mixture of carrier acids in 1 ml of ether-methanol (1:1 v/v) was added. Saponification was complete after samples were allowed to stand overnight at room temperature or were refluxed for 45 min.

Saponified samples were acidified with 12 N HCl and with a small amount of water and methanol were transferred to 500 ml separatory funnels. Sufficient chloroform-methanol (2:1 v/v) was added (12) to give a single phase (ca. 150 ml). After 30 min 150 ml of saturated NaCl solution was added; the chloroform phase was then removed and the aqueous phase was re-extracted with 50 ml of chloroform. The combined chloroform extracts were washed twice with 150 ml of water, dried over anhydrous Na_2SO_4 and evaporated on a rotary evaporator. The residual fatty acid samples were then treated with freshly prepared diazomethane (13). A portion of the resulting methyl esters was dissolved in toluene scintillator [4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-2-(5-phenyloxazoyl)-benzene per liter of toluene] and radioactivity was measured with a liquid scintillation spectrometer (Packard).

A portion of the methyl esters was separated into saturated, monoene and polyene fractions (14) by thin layer chromatography (TLC) on

TABLE III

Chain Lengths of Dicarboxylic Acid Fragments Obtained After Oxidation of Isolated Monoenoic Esters[a]

Dicarboxylic acid chain length (no. of carbons)	Radioactivity in dicarboxylic acid after oxidation, %		
	18:1 from 1-^{14}C-stearic	16:1 from 1-^{14}C-palmitic	14:1 from 1-^{14}C-myristic
5	0.3	0.9	1.8
6	0.6	ND[b]	0.4
7	0.4	2.4	5.9
8	4.9	4.1	2.4
9	86.3	80.2	82.5
10	4.0	10.4	1.7
11	3.5	2.0	5.3

[a]The monoenoic fatty acids obtained from action of the 22,000 X *g* supernatant fraction upon saturated 1-^{14}C-acids were isolated by TLC ($AgNO_3$) and GLC and subjected to permanganate-periodate oxidation. Isolated dicarboxylic acids were separated by GLC, and the effluent fractions were collected and subjected to scintillation counting.

[b]ND, not determined.

Silica Gel G-silver nitrate (90:10) with chloroform-acetic acid (99.5:0.5). The fractions, visible under ultraviolet light after spraying with 2,7-dichlorofluorescein, were scraped from the plates and eluted with ether. The extracts were evaporated in scintillation vials under nitrogen, fluid was added and radioactivity determined. Average recovery of initial fatty acid label was 75% for oleic acid, 90% for saturated acids.

Location of the Double Bond

The position of the double bond in the monounsaturated acids was determined by periodate-permanganate oxidation as described by von Rudloff (15). Esters were first converted to the free acids by refluxing 1 hr with 5% KOH in 95% methanol, acidification and ether extraction. After oxidation, the reaction mixture was acidified, carrier mono- and dicarboxylic acids added, the acids extracted with three volumes of ether, and the ether extracts washed with saturated sodium chloride solution. The ether phase was dried with sodium sulfate, evaporated, and mono- and dicarboxylic acids separated by TLC (16) on rhodamine impregnated Silica Gel G plates with hexane-ether-acetic acid (70:30:1). After removal and extraction from the adsorbent, fractions were assayed for radioactivity or esterified and analyzed by gas liquid chromatography (GLC).

For GLC, glass columns (¼ in. O.D. x 10 ft) packed with 20% diethyleneglycol adipate polyester on 60/80 mesh Chromosorb W and conditioned 24 hr at 200 C were operated at 180 C for dicarboxylic esters and at 205-210 C for long chain fatty acid esters; flask vaporizer and injection port, 300 C; detector block 250 C; helium flow rate, 60-70 ml/min at exit port. Instrument was Aerograph 90, thermal conductivity unit with Aerograph 12-volt DC constant power supply to maintain filament current at 150 ma and Sargent SR recorder. Effluent collections were manual (9,17) into scintillation fluid.

Decarboxylations were essentially as described by Brady et al. (18). The reactions proceeded in 25 ml Erlenmeyer flasks with shell vials inserted and serum caps wired tightly.

TABLE IV

Products Obtained From Incubation of 1-^{14}C-Oleic Acid With a Soluble Fraction (105,000 x *g* Supernatant) From Cotyledons[a]

Incubation time, min	Radioactivity recovered in isolated fatty acid fractions, %			Radioactivity from polyene fragments as dicarboxy acids, %
	Saturated	Monoene	Polyene	
30	0.4	93.8	5.8	94.5
60	0.7	83.5	15.8	95.5
120	1.1	74.5	24.6	94.6

[a]Experimental conditions identical to those in Table I.

TABLE V

Alterations in Chain Length of Labeled Fatty Acids During 3 Hr Incubation With 22,000 x *g* Supernatant Fraction[a]

1-^{14}C-Substrate	Per cent of recovered counts in lipid after incubation					
	C12	C14	C16	C18	C20	C22
14:0	0.6	92.7	1.3	2.5	2.2	0.2
16:0	0.1	0.2	91.7	4.4	2.6	0.9
18:0	---	0.2	1.0	97.2	1.6	---
18:1	---	---	1.0	96.1	2.9	---

[a]Incubation conditions were the same as for Table I, with addition of 1 μmole of acetyl CoA. Separations of fatty acids were performed as described in Table III.

After reaction, flasks were cooled, CO_2 was trapped as described above, the contents of the vial transferred to a scintillation vial for counting and the remaining contents of the reaction flask made alkaline and extracted with ether to obtain the fatty amine (19).

RESULTS AND DISCUSSION

Desaturation of 1-^{14}C-Fatty Acids

When labeled C_{12} to C_{18} saturated acids were complexed with albumin and incubated with the supernatant fractions (22,000 x *g* or 105,000 x *g*) from cotyledons, up to 19% of the recovered radioactivity was in the monoenoic and polyenoic fatty acid fractions (Table I). Most of the conversion occurred within the first hour. At all times radioactivity in the monoenoic fraction exceeded that of the polyenoic fraction.

To ascertain that desaturation rather than fragmentation and resynthesis had occurred, portions of the monoenoic fractions were treated with periodate-permanganate. The dicarboxylic acid fragments (Table I) which were then isolated generally contained more than 90% of the recovered label. The monoenoic products from lauric acid were a notable exception, indicating that some degradation and resynthesis had occurred during its incubation with the supernatant fraction. Additional evidence that the original carbon chain remained intact was seen in the results of Schmidt decarboxylation of the palmitoleate isolated after incubation of 1-^{14}C-palmitate (Table II). More than 80% of the label was recovered as $^{14}CO_2$ from the 1 position with as little as 2.3% remaining in the amine fragment.

It is evident, therefore, that the supernatant fraction catalyzes direct desaturation of long-chained saturated fatty acids. This action was reduced by more than 50% when incubations were performed under nitrogen. The reaction may be catalyzed by an oxidative desaturase similar to that present in animal tissues (6) and certain microorganisms (5).

Position of the Unsaturated Linkage

When monoenoic acids produced from three 1-^{14}C-saturated acids in a supernatant fraction were separated and oxidized, the distribution of radioactivity in the dicarboxylic esters revealed that the major labeled fragment was azelaic acid (Table III).

The desaturatase system is quite specific for the 9,10 position, irrespective of the chain length of the fatty acid. This represents a further similarity to desaturase systems of animals (6), yeast (7) and *Chlorella* (20).

Further Desaturation and Chain Elongation

The 105,000 x *g* supernatant fraction also acted upon 1-^{14}C-oleate to effect further desaturation (Table IV). Nearly 25% of the recovered label after 2 hr was in the polyene fraction when separated by TLC and determined by assay for radioactivity. On oxidation of this polyene fraction with periodate-permanganate nearly 95% of the recovered label was in the dicarboxylic acid fraction. It was evident, therefore, that the chief reaction products were polyunsaturated fatty acids. This was confirmed upon demonstration by GLC that the dicarboxylic fraction was preponderantly C_9 and by Schmidt decarboxylation that nearly all of the isotopic carbon in the polyenoic fraction was recoverable in the CO_2. Fractions from safflower seedlings also have been shown to desaturate oleic acid (21,22).

To observe whether chain elongation of added labeled fatty acids accompanied desaturation 1 μmole of non-labeled acetyl CoA was added to the incubation mixture with each of four substrates (Table V). Clear evidence for chain elongation was observed with all the acids. Resynthesis after partial degradation of

fatty acids was minimal.

The results of these experiments demonstrate that (a) plant systems other than intact chloroplasts (8) can perform direct aerobic desaturation of palmitate and stearate; (b) the system contained in soybean cotyledons which performs this desaturation is present in both the microsomal and supernatant fractions; and (c) these fractions which produce monoenoic fatty acids also contain systems which effect further desaturation to polyunsaturated compounds as well as elongation of carbon chains, actions which are characteristic of the intact cotyledon cells.

It is not yet clear whether the palmitate-stearate desaturatase system is completely soluble or occurs in the 105,000 x *g* supernatant fraction as finely fragmented particulate material. It is also not known whether ferredoxin and acyl carrier protein (ACP) are active components of the cotyledon system. The demonstration that stearyl ACP is desaturated by a 105,000 x *g* supernatant fraction from *Euglena* (23) and by spinach chloroplasts in the presence of ferredoxin (8) suggests that these substances may be required. If so, they are retained in the 105,000 x *g* supernatant fraction from the cotyledons.

No explanation is apparent as to why avocado (4) and various other plant materials have failed to yield palmitate-stearate desaturating systems. In our earlier studies with the soybean cotyledon we observed substantial differences in level of activity in mature versus nearly mature cotyledons. Similar effects of maturity have been demonstrated with ricinoleic acid formation by castor bean preparations (24).

The demonstration that direct aerobic desaturation occurs in subcellular fractions from soybean cotyledons raises the question whether this is the main pathway for the formation of oleic acid in the intact plant. Failure to demonstrate the existence of such systems (4) led to the postulate that plants produce oleic acid from shorter-chained acids by some route other than direct desaturation of palmitic and stearic acids. This view was generally accepted by others (5) but recently had been altered (8). Evidence for specific intermediates in such a pathway is lacking in plants. For soybean cotyledons the concept of an alternative route appears to be dispensable. Argument against direct desaturation of palmitate and stearate as the main route to oleate in plants finds support in observations of a relatively low specific activity found in stearic acid as compared to that in oleic acid. The presence of enzyme-bound intermediates, in which stearate is rapidly converted to oleate, with a much slower equilibrium between the enzyme-bound stearate and free stearic acid has been postulated by James (25). Differences in rates of exchange between free and enzyme-bound forms of the fatty acids and the consequent differences in size of the different active pools as against inactive pools may account for the apparent discrepancies in specific activities. It is hoped that some of these points will be clarified in further work.

ACKNOWLEDGMENT

Supported in part by Research Grant No. A-4778 of the National Institutes of Public Health.

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[Received December 2, 1968]

The Effect of Aging of Human Red Cells in Vivo on Their Fatty Acid Composition

GERALD B. PHILLIPS[1], JAMES T. DODGE[2] and CALDERON HOWE, Department of Medicine, The Roosevelt Hospital, and the Departments of Medicine and Microbiology, College of Physicians and Surgeons, Columbia University, New York, New York 10019

ABSTRACT

Using improved methods for fatty acid analysis and a density gradient method for separation of red cells according to age, we analyzed the fatty acid composition of a young fraction and an old fraction of red cells from each of seven normal subjects and from one patient with polycythemia vera. Twenty-four fatty acid peaks were quantified. The individual phospholipid composition of both fractions was determined in the red cells of two normal subjects. Statistically significant changes in the relative amounts of major fatty acids were observed with red cell aging. Furthermore, a pattern of change was evident in that the relative amounts of four of the five fatty acids of C_{18} or shorter chain length showed an increase and all 19 fatty acids of C_{20} or longer chain length showed a decrease with red cell aging. While the change with aging in every fatty acid peak was not statistically significant, the consistency in the direction of the change in the fatty acids of C_{20} or longer chain length was highly significant. A suggested change in the distribution of the major phospholipid groups with red cell aging did not appear to explain completely the changes in fatty acids.

INTRODUCTION

The easy accessibility of red cells and the availability of methods for their separation according to age (1-5) make the red cell particularly suitable to a study of the aging in vivo of a cell in man. Since essentially all of the lipid of the human red cell resides in its membrane (6,7) and constitutes about 35% to 45% of the dry weight of the membrane (6), a study of the red cell lipid in relation to age provides information on the aging in vivo of a cell membrane. In 1958, Prankerd (8) demonstrated that the phospholipid and unesterified cholesterol content of the human red cell decreased with age, a finding that has been confirmed (9,10). Munn (11) reported that the linoleic acid content of the human red cell increased with age; this observation has been corroborated by van Gastel et al. (10) and by reports of decreased red cell linoleic acid levels in association with reticulocytosis (12,13). Reports of changes in other fatty acid levels have been conflicting. The distribution of the individual human red cell phospholipids has been observed to remain constant during aging in vivo (9,10,13).

The recent development of improved methods of gas liquid (GLC) and thin layer chromatography (TLC) allows a more detailed and reliable analysis of human red cell fatty acids than had previously been performed (14). The present study, therefore, was carried out to determine the fatty acid composition of young and old red cells of individual subjects. Significant changes in major fatty acids in addition to linoleic acid were observed with aging, and a pattern of change in the fatty acid composition emerged.

MATERIALS AND METHODS

A 35 ml sample of blood was obtained by venapuncture from each of seven apparently healthy non-fasting subjects, five male and two female, between the ages of 21 and 49; six of the samples were collected into Vacutainer tubes (Becton, Dickenson and Co., Rutherford, N.J.) containing 0.75 ml of a 30% solution of EDTA; the 7th was taken in Alsever's solution (15). Each sample was processed immediately after collection. Venous blood from a patient with polycythemia vera was collected in ACD Solution (USP Formula A) and stored for 4 days at 4 C before analysis. The phthalic acid ester method of Danon and Marikovsky (5) was used for density gradient separation of the red cells. On the basis of preliminary runs with capillary tubes, we selected phthalate oil of density (D) 1.110 for separation of the older (lower) cells and D 1.098 for the younger (upper) cells, either fraction representing approximately 5% to 10% of the total packed red cell volume. The cells and oil were put in 1 ml plastic tuberculin syringes (Becton, Dickenson and Co., Rutherford, N.J.) 4.0 mm I.D., 7.0 mm O.D. 7.0 cm long, the outlets of which had been sealed off by heating. These tubes were spun in an Adams Microchemistry Centrifuge CT-3000 (Clay-Adams, Inc., New York, N.Y.) at 15,000 x *g* for

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[1] Recipient of Lederle Medical Faculty Award.

[2] This work was done during the tenure of an Advanced Research Fellowship of the American Heart Association.

15 min. In 2 samples (one with Alsever's solution), each oil was layered on top of the red cells, which were then spun to obtain the young and old fractions. In one of these 2 samples (collected in EDTA), the plasma and buffy coat were removed and the cells were washed once with an equal volume of isotonic saline prior to density gradient separation. The other 5 normal samples and the polycythemic sample were centrifuged to remove most of the plasma and buffy coat. The remaining packed cells were mixed with 0.2 vol of plasma and the cells were separated according to age as follows. Cells were layered on top of D 1.110 oil in tuberculin syringes and spun to obtain the lower layer. The upper cells were pooled and then added on top of D 1.098 oil and spun to obtain the upper layer. The upper layers were pooled, added on top of D 1.094 oil, and spun; as a result, all of the red cells passed through the oil to the bottom, leaving the buffy coat and plasma on top of the oil. The foregoing procedure was useful not only to remove the buffy coat but to permit both the upper and lower sample to go through the oil once, so that treatment of both samples with respect to the oil was comparable. Despite the differences in the anticoagulant used and in separation procedures, all of the samples showed similar individual fatty acid differences between the older and younger cells.

As a check on the age separation, the osmotic fragility of the fractions obtained from one sample was measured (4) and was found to be greater in the old than in the young cells. In addition, reticulocyte counts of the young fractions of 3 samples were each 4% to 5%, while no reticulocytes were found in the old fractions. In another sample, measurements were made of glucose-6-phosphate dehydrogenase activity, pyruvate kinase activity and reticulocytes, and found to be 11.2 (μM TPN converted to TPNH per minute per gram hemoglobin), 2.6 (μM DPNH converted to DPN per minute per 10^{10} red cells), and 4% in the young fraction as compared to 5.4, 0.85 and 0%, respectively, in the old fraction. No white cells were found in the young or old fractions of this sample. Other investigators (16,17) have also provided evidence to support the validity of the separation method (5) used in this study.

To determine whether the phthalate removed any of the red cell lipid, we centrifuged about 4 ml of red cells through 0.5 ml of the phthalate. The phthalate was then dissolved in hexane and chromatographed on a column of 1 gm silicic acid using 10 ml hexane-diethyl ether (9:1 v/v), to elute the phthalate and any cholesterol present, and 7 ml methanol to elute any phospholipid. Each eluate was concentrated by evaporation and put on a silicic acid thin layer plate for chromatography. The hexane-diethyl ether eluate was developed with hexane-diethyl ether (70:30 v/v) to separate phthalate from cholesterol, and the methanol eluate was developed in chloroform-methanol-glacial acetic acid-water (25:15:4:2 v/v) (18) for separation of phospholipids. No evidence of cholesterol or phospholipid was found in the phthalate through which the erythrocytes had been passed even though 1 μg of these substances can be detected by TLC.

After density gradient separation, all of the samples were similarly treated. The red cells were washed with approximately 10 vol of isotonic saline 3 times. Slight to moderate hemolysis (estimated to involve about 1% of the red cells) was usually evident on the first washing, and more often with the lower cells than with the upper; the extent of hemolysis decreased on the subsequent 2 washings. To prepare red cell fatty acid methyl esters (FAME) for GLC, we isolated the fatty acids by exposure of the red cells to 2 N HCl for 18-20 hr and extraction of the fatty acids with pentane, and converted them to the methyl esters by the method of Morrison and Smith (19). The details of this procedure have been described previously (20). GLC of the FAME was carried out with a Barber-Colman instrument, model 5000, equipped with 8 ft columns of EGSS-X 8% on Gas Chrom P, 100/120 mesh (Applied Science Laboratories, Inc., State College, Pa). The methods for GLC and identification and quantitation of the peaks have been described previously (14).

The 2-thiobarbituric acid (TBA) test was performed on the red cells as described previously (21).

RESULTS

The mean distribution and range between subjects of the individual fatty acids of the young and old red cells of the 7 normal subjects are shown in Table I. The values for the patient with polycythemia vera are shown separately in Table I. Using the "t" test on paired samples of young and old cells (6 degrees of freedom) as a statistical criterion of significance, we found differences in the relative amounts of several fatty acids between young and old cells. The levels of palmitic (16:0), oleic (18:1ω9) and linoleic (18:2ω6) acids were higher, while arachidonic acid (20:4ω6), 20:3ω6 + 22:0, and 22:4ω6 + 24:1ω9 were lower in the older cells. (In this abbreviation of the fatty acids, the first 2 digits state the number of carbon atoms, the

TABLE I

Fatty Acid Composition of Young and Old Human Red Cells[a]

Component[b]	Young red cells			Old red cells			Per cent change with aging (normals)	p[c]
	Normal subjects		Poly-cythemic	Normal subjects		Poly-cythemic		
	Mean	Range g/100 g fatty acid		Mean	Range g/100 g fatty acid			
16:0	19.3	17.9-20.6	19.7	20.7	18.8-21.9	23.4	+7	<0.001
17:0	0.48	0.33-0.62	0.42	0.58	0.49-0.70	1.1		
18:0	15.9	14.9-18.1	16.3	15.5	14.3-18.3	14.9		
18:1ω9	12.8	12.0-13.9	12.3	14.3	13.2-15.7	15.8	+12	<0.001
18:2ω6[d]	8.3	6.0-10.8	6.0	10.1	7.5-13.9	8.5	+22	<0.01
20:0	0.44	0.26-0.71	0.36	0.42	0.32-0.58	0.43		
20:1ω9 + 18:3ω3[e]	0.45	0.19-0.60	0.31	0.29	0.17-0.46	0.43		
20:2[f] + 21:0[e]	0.46	0.18-1.0	0.38	0.28	0.10-0.45	0.34		
20:3ω9	0.15	0.00-0.38	0.38	0.11	0.00-0.24	0.28		
20:3ω6 + 22:0	3.4	2.9-4.2	2.9	3.1	2.5-3.8	2.9	-9	0.02
20:3ω6[g]	1.3	1.1-1.6	0.85	1.1	0.84-1.5	1.1		
22:0[g]	2.2	1.8-2.6	2.1	2.0	1.7-2.3	1.8		
20:4ω6 + 22:1ω9[h]	16.2	14.7-17.6	19.8	14.2	12.6-16.1	14.6	-12	<0.001
23:0 + 20:4ω3[e]	0.54	0.33-0.88	1.3	0.47	0.32-0.67	0.85		
20:5ω3 + 22:3ω9[e] + 22:2[f]	0.45	0.22-0.82	0.45	0.42	0.18-0.69	0.34		
24:0	4.9	3.3-5.6	4.5	4.7	3.9-5.4	3.8		
22:4ω6 + 24:1ω9	8.2	7.3-9.8	8.3	7.4	6.4-9.2	6.0	-10	<0.01
22:4ω6[i]	2.9	2.4-3.6	2.9	2.6	1.9-3.6	1.7		
24:1ω9[i]	5.3	4.9-6.2	5.4	4.9	4.5-5.6	4.4		
22:5ω6	0.73	0.42-1.0	1.3	0.65	0.44-0.88	1.0		
24:2[f]	0.45	0.28-0.82	0.48	0.44	0.26-0.79	0.55		
22:5ω3	2.1	1.6-2.8	1.8	1.9	1.4-2.4	1.6		
22:6ω3 + 26:0[j]	4.5	2.9-6.7	3.0	4.2	2.6-5.7	2.9		
26:1ω9 + 24:4ω6[e]	0.41	0.35-0.51	0.17	0.35	0.14-0.55	0.32		

[a]Values obtained on seven normal subjects and 1 patient with polycythemia vera. Mean and range values are on the seven normal subjects.

[b]This shorthand designation for fatty acids is explained in the text.

[c]P, Probability that the value for young and old red cells of the normal subjects is the same; average difference between the young and old cells on paired samples was tested for significance by "t" test (six degrees of freedom); p values shown only where significant.

[d]May contain 18:2ω9. The fatty acids are listed in order of elution from the column.

[e]Trace component, $<0.05\%$ of the total.

[f]Contains ω9 and ω6 components.

[g]This peak could be quantified in six subjects.

[h]Makes up about 2% of this peak in normal human red cells (14).

[i]This peak could be quantified in five subjects.

[j]Makes up about 5% of this peak in normal human red cells (14).

third digit states the number of double bonds, and the digit after the omega states the number of carbon atoms from the methyl end of the acyl chain to the middle of the nearest double bond). Differences in other fatty acids may have been present but not statistically significant because of the small sample size. This possibility is suggested by the values for the red cells from the patient with polycythemia, where the differences almost always were in the same direction but more exaggerated than in the red cells from the normal subjects.

Comparison of the fatty acid values of young and old cells discloses a striking pattern, as shown in Table I. Whereas the values for 4 of the first 5 fatty acid peaks listed were higher in the old than in the young cells, the values for all of the 19 subsequent peaks were higher in the young than in the old cells. Although the difference in values for most of the peaks is not statistically significant, the consistency in the direction of change in the peaks listed after 18:2ω6 is highly significant. Since the peaks shown in Table I are listed in order of elution from the chromatographic column, the fatty acid changes seen with red cell aging may be a function of the physical properties of the fatty acids related to their relative binding affinity for the column. The change in direction of the differences between 18:2ω6 and 20:0 also suggests the possibility that chain length may be the critical factor involved.

In Table II, the values for the fatty acid peaks that showed significant age differences are compared in the young, old and native (i.e., not subjected to the separation procedure) red cells of two normal subjects. The observation that the values for the native red cells fall between those of the young and old cells in 5 of the peaks suggests that the young or the old fraction was not a population of cells artifactually produced by the procedure.

Table III shows the age distribution of the individual phospholipids and of the age-dependent fatty acids (Table I) of the red cells of 2 normal subjects. Significant difference in distribution of individual phospholipids was not apparent in the red cells of Subject H. L. Analysis of the red cells of Subject D. S., however, suggested that the young cells had a higher relative and absolute amount of phosphatidyl serine and a lower relative amount of lecithin and sphingomyelin than the old cells; the intermediate values for these phospholipids in the fraction of intermediate age supported this finding. The small age differences observed in the red cells of Subject H. L. may have been due to the relatively poorer density gradient separation of this sample owing to the large

TABLE II

Fatty Acid Composition of Young, Old and Whole Samples of Red Cells of Two Normal Subjects[a]

Component[b]	Young	Whole	Old
	g/100 g fatty acid		
16:0	18.5	19.0	19.6
18:1ω9	13.3	14.4	14.9
18:2ω6	7.0	8.6	8.8
20:3ω6 + 22:0	3.6	3.2	3.0
20:4ω6	16.3	15.4	14.3
22:4ω6 + 24:1ω9	8.7	8.2	8.4

[a]Whole refers to samples of the native red cells before separation of the age fractions. Data shown are mean values from two normal subjects of the fatty acids which showed significant age differences (Table I).

[b]This shorthand designation for fatty acids is explained in the text.

fractions taken for the young and old cells; the fatty acid levels in the red cells of this subject are consistent with the difference in mean age between the 2 fractions being considerably less than in Subject D. S. Nevertheless, in Subject H. L. a significant difference in the level of linoleic acid was evident with little, if any, difference in the level of lecithin, where most of the red cell linoleic acid resides (14). That a change in lecithin level was not responsible for the change in linoleic acid level is corroborated by the observation that the lecithin of the old cells in this subject had a linoleic acid value of 21.1% compared with a value of 15.0% in the lecithin of the young cells. Similarly, although the relative amount of sphingomyelin may be higher in the old cells (Subject D. S.), the relative amounts of 24:0 and 24:1, which in human red cells are found almost exclusively in the sphingomyelin (14), were higher in the young cells. The significantly higher level of 24:1 found in the sphingomyelin of the young cells than of the old cells in Subject H. L. supports this observation. Thus, it appears that the differences in fatty acid composition between young and old cells cannot be explained entirely by a difference in distribution of the major phospholipid groups.

The intermediate fatty acid values found in the red cell fraction remaining after the removal of the young and old fractions in Subject D. S. (Table III) indicates that the age differences were not confined to a small population of young (reticulocytes) or old cells and suggests that the age changes in fatty acid composition may be a continuous process, although the pattern of change for individual fatty acids may be different.

TABLE III

Phospholipid and Fatty Acid Composition of Red Cells of Two Normal Subjects[a]

Subject	H. L. Young %	H. L. Old %	D. S. Young %	D. S. Middle[b] %	D. S. Old %
Phospholipid distribution					
Phosphatidyl ethanolamine	26.4	26.4	29.7	42.1	29.0
Phosphatidyl serine	15.0	14.2	15.5		12.2
Phosphatidyl inositol[c]	0.5	0.4	0.8	0.7	0.6
Lecithin	29.7	30.4	28.8	30.2	31.3
Sphingomyelin	26.3	26.2	23.4	24.4	25.3
Lysolecithin	1.6	1.4	1.5	1.9	1.2
Origin on TLC plate	0.6	1.0	0.4	0.6	0.4
Recovery of P after TLC	98	102	99	96	99
Lipid P-μmoles/ml red cells	3.94	3.88	4.62		4.15
Fatty acid[d] distribution					
16:0	19.5	18.3	20.5	20.8	21.9
18:1ω9	11.9	12.0	12.5	13.5	14.4
18:2ω6	8.7	9.8	7.5	9.0	9.9
20:3ω6 + 22:0	4.0	3.3	3.3	2.8	3.3
20:4ω6	16.9	16.0	16.4	16.3	14.9
22:4ω6 + 24:1ω9	7.6	7.3	8.2	7.6	7.0

[a]Phospholipid distribution was determined five times on a sample of young and old red cells in Subject H. L. and four times in Subject D. S.; the middle sample was done in duplicate.

[b]Red cell sample remaining after removal of the young and old red cell fractions.

[c]Not positively identified.

[d]This shorthand designation for fatty acids is explained in the text.

The decrease in the relative amount of arachidonic and other polyunsaturated fatty acids with red cell aging suggested autoxidation, to which these fatty acids are particularly susceptible (22), as a possible cause of this decrease. The results of the TBA reaction, a measure of lipid autoxidation, were compared in the young and old red cells of two of the normal subjects. In one subject, the young cells showed 0.7 and the old 0.8 moles x 10^{-8} malonaldehyde per milliliter red cells, and in the other subject the corresponding values were 0.9 and 0.9 (normal range for native red cells was 0.7 to 1.3). Thus, no significant difference in the TBA reaction between the young and old cells of either subject was evident and all values fell within the normal range.

DISCUSSION

With the use of improved methods for fatty acid analysis in the present study, we have provided evidence for a statistically significant change in the relative amount of several major fatty acids of human red cells with aging in vivo in addition to the increase in linoleic acid reported by others (10-13). These findings also confirm the decrease in the relative amount of arachidonic acid with human red cell aging noted by van Gastel et al. (10). Furthermore, the present study shows a striking change in the pattern of fatty acid composition with red cell aging characterized by an increase in the relative amounts of fatty acids of 18 carbon chain length (C_{18}) or less, except for stearic, and a decrease in the relative amounts of fatty acids of 20 carbon chain length (C_{20}) or more. Thus, the relative amounts of most if not all of the fatty acids of human red cells appear to be affected by the aging process. Walker and Yurkowski (23) also demonstrated changes in the relative amounts of fatty acids with aging of rat red cells in vivo and showed that the direction of change of a given fatty acid was dependent on the dietary lipid intake.

Since almost all of the fatty acid of human red cells is found in the phospholipid (24), and each major phospholipid has a characteristic fatty acid composition, we analyzed the phospholipids of the red cells of 2 of the subjects in order to determine if the fatty acid changes reflected primary changes in distribution of the individual phospholipid groups. The 3 fatty acids that were higher in the old cells, 16:0, 18:1ω9 and 18:2ω9, and the one that probably was higher, 17:0, are the only 4 fatty acids of human red cell phospholipids that have their highest concentration in lecithin and make up about 75% of the lecithin fatty acids (14). In addition, the relative amounts of the fatty acids of 20 carbon chain length or more are essentially all lower in the lecithin than in the

cephalin of human red cells (14). Therefore, an increase in the relative amount of lecithin with red cell aging could explain the fatty acid changes found. The results of the analysis did indeed suggest an increase in the relative amount of red cell lecithin and sphingomyelin and a decrease in the relative and absolute amount of cephalin, especially phosphatidyl serine, with aging. Small but statistically insignificant changes in lecithin, sphingomyelin and cephalin in the same direction were also noted by van Gastel et al. (10). Nevertheless, the suggested change in the distribution of the major individual phospholipid groups in the present study did not appear to be sufficient to account for the fatty acid changes found. Differences between old and young cells in the fatty acid composition of both lecithin and sphingomyelin in the one sample analyzed supported this observation. Similar conclusions have been reported by others (9,10,13).

Although the mechanism of the change in fatty acid composition of human red cells with aging in vivo is not known, the following explanations may be considered: (a) exchange of phospholipids between red cells and plasma (25-27); the direction of change of certain fatty acids in the present study, however, does not appear to be consistent with this explanation; (b) an alteration in binding affinity of red cells for phospholipids with aging; (c) selective loss of phospholipids with maturation of the reticulocyte; and (d) an alteration in red cell transacylating activities (28-30) with aging. Since the transacylation system appears to play an important role in determining the fatty acid composition of the β position of human red cell lecithin (31), its alteration with aging could account for much if not all of the changes observed in the present study. The possibility that the above mechanisms are operating simultaneously or that an entirely separate mechanism is responsible for the age changes in red cell fatty acids described must also be considered.

ACKNOWLEDGMENT

This investigation was supported by U.S. Public Health Service Grants HE 02907, HE 10950 and AI 03168. Measurements of red cell enzymes and of reticulocytes were made in the laboratory of C. Grossbard, Department of Medicine, Columbia University, N.Y.C.

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[Received February 10, 1969]

Gas Liquid Chromatographic Analysis of Sphingosine Bases in Sphingolipids of Human Normal and Multiple Sclerosis Cerebral White Matter[1]

EZIO A. MOSCATELLI and **ELIZABETH ISAACSON**, Biochemistry Department,
The University of Texas Southwestern Medical School at Dallas, Texas 75235

ABSTRACT

Sphingomyelins, cerebrosides and sulfatides were isolated from white matter of human normal and multiple sclerotic brain. Column chromatography was used for class separations, which were monitored by thin layer chromatography. Acid-catalyzed methanolysis, followed by isolation of bases and gas liquid chromatography of periodate-produced aldehydes, was used to determine sphingosine base composition of each sphingolipid. Reflux methanolysis (where HCl = 2 N) was used for cerebrosides and sulfatides, but could not be used for reliable sphingomyelin base analysis, for which sealed-tube methanolysis at 70-80 C (where HCl = 1 N) was used. Our results suggest there is little, if any, difference in sphingosine base chain-length distribution among the sphingolipids analyzed.

INTRODUCTION

The chemical pathology of multiple sclerosis (MS) has drawn the attention of a number of investigators in the last decade. Some of the work on this demyelinating disease has concerned various fractions of blood (1-4), but the most significant work has been done on central nerve tissue (5-7), especially on myelin itself (8,9). In fact, Kishimoto et al. (7) suggested that the chemical pathology in MS appears to be confined entirely to the myelin sheath.

Several years ago, O'Brien (10) demonstrated a seven to tenfold deficiency of sphingolipids containing long chain fatty acids in white matter from metachromatic leukodystrophy, a sulfatide storage disease in which demyelination occurs. Similar deficiencies were reported by Svennerholm (11) in several other myelin diseases, while Gerstl et al. (12) noted this phenomenon in MS itself. These observations, together with the critical importance to myelin integrity of hydrophobic bonding between long alkyl chains (13,14), led O'Brien to suggest (10,15) that a deficiency of long chain sphingolipid molecules might lead to such instability between concentric myelin layers as to explain many, if not all, demyelinating diseases. The application of this hypothesis to metachromatic leukodystrophy was disputed by Stallberg-Stenhagen and Svennerholm (16), who suggested an alternative explanation for O'Brien's data. The basic premise, however, remains sound, i.e., a significant deficiency of long alkyl chains would be expected to lead to less interdigitation in the myelin lipid double layer, which in turn might well lead to demyelination. Since nonganglioside sphingolipids form a significant part of the complex lipid content of myelin, we considered it of interest, as well as a further test of O'Brien's hypothesis, to determine and compare the alkyl chain length distribution of the sphingosine moiety of sphingomyelins, cerebrosides and sulfatides in normal and in MS white matter. The results of a control experiment, which showed no difference in polar lipid sphingosine base composition between normal myelin and white matter, are also reported.

Gas liquid chromatography (GLC) of periodate-produced aldehyde derivatives of sphingosine bases, which were liberated from sphingolipids by acid-catalyzed methanolysis, was used to analyze sphingosine base composition, because this has proven to be a reasonably reliable method for the determination of sphingosine base alkyl chain length isomers (17,18). Reflux methanolysis of isolated sphingomyelins gave rise to difficulties, which will be discussed below.

EXPERIMENTAL PROCEDURES

Preparation of White Matter and Myelin

Normal human white matter was obtained by blunt dissection of adult corpus callosum. Autopsies performed within 12 hr after death revealed no decomposition or neurological disorder. The corpus callosum was removed at autopsy and immediately frozen for storage until use. Three such normal corpus callosums were used for this study. Only one MS brain section was available. MS white matter consisted of a portion of corpus callosum and adjacent white matter at the superior lateral aspect of the lateral ventricle. This was carefully dissected out to include at least one

[1]Presented in part at the AOCS Meeting, New York City, October, 1968.

TABLE I

Aldehydes Derived From Sphingosine Bases

Sphingosine base	Aldehyde	*Relative retention time*[a]
16:Dihydrosphingosine[b]	Tetradecanal	0.47
18:Dihydrosphingosine	Hexadecanal	1.00
18:Sphingosine	Hexadecenal	2.07
20:Sphingosine	Octadecenal	4.26
---	---	5.00[c]
O-Methyl 18:sphingosine[d]	O-Methyl hexadecenal	6.21

[a]Calculated at 140 C, relative to hexadecanal, which arbitrarily equals 1.00.

[b]For the sake of clarity, conventional sphingosine base designations are used, rather than the less familiar, much longer nomenclature recommended by the Commission on Biochemical Nomenclature of IUPAC and IUB.

[c]The peak at RRT of 5.00 is an aldehyde we have not yet identified.

[d]This is probably the 5-0-methyl isomer (see text).

clearly demarcated plaque, as well as several less clearly defined ones. Plaques were estimated to represent between 5% and 10% of the MS white matter. Dissection was performed on a thawed section of adult MS brain which had been stored frozen for several months, but had not been autopsied until 72 hr after death.

Myelin was prepared from normal corpus callosum according to the ultracentrifugal method of Autilio et al. (19), but not further fractionated into light and heavy myelin, inasmuch as this distinction may well be artifactual. A portion of myelin, prepared from the same corpus callosum, was dispersed in distilled water and lyophilized.

Isolation of Sphingolipids

Total polar lipids, less gangliosides, were prepared by Folch extraction and wash (20), followed by silicic acid column separation, as previously described (18).

Sphingolipids were isolated from polar lipids by column chromatography on activated, acid-washed silicic acid, 100-200 mesh (Unisil, Clarkson Chemical Co., Williamsport, Pa.) using modifications of the method of Wells and Dittmer (21). In these experiments we found it unnecessary to perform preliminary deacylation of alkali-labile phospholipids. Column separations were monitored by analytical thin layer chromatography (TLC), and crude sphingomyelin, cerebroside and sulfatide fractions were prepared by combining appropriate column cuts. Cerebrosides and sulfatides were subsequently purified by chromatography on small Florisil columns, while a small Alumina column was used to purify normal sphingomyelin. MS sphingomyelin was not subjected to Alumina column purification because it was available in smaller quantity and was already free of cerebrosides and sulfatides, as a Unisil eluate. These were checked for purity by analytical TLC, using two solvent systems.

Methanolysis Methods

Two methanolysis methods were used to liberate sphingosine bases from sphingolipids. For cerebrosides, sulfatides and polar lipid mixtures, the reflux method of Sweeley and Moscatelli (17) was used. This consisted of refluxing approximately 10 to 40 mg of lipid, under nitrogen, for 5 1/2 hr in 15 ml of methanol: conc. HCl, 80:16. In the case of sphingomyelin, the reflux method gave such variable results, together with production of intolerable levels of artifacts, that the newer method of Gaver and Sweeley (27) was used. In this method, approximately 2 to 10 mg of sphingomyelins were heated under nitrogen in a Teflon-lined screw-capped tube at 70-80 C for 18 hr in methanol-conc. HCl-water, 82:8.6:9.4. The basic difference in these two methanolysis mixtures is that the former is 2 N, and the latter 1 N with respect to HCl.

Isolation of Bases and Preparation of Aldehydes

Bases were isolated as previously described (18), with the minor exception that hexane, rather than petroleum ether, was used for initial removal of fatty acid methyl esters from the acidic methanolysate. Periodate oxidations were performed, using $NaIO_4$ as previously reported (18), or by the variation described by Carter and Hirschberg (30). We began these studies using the latter, but later returned to the more convenient former method, when, in a control experiment, they gave essentially identical results.

Thin Layer Chromatographic Analyses

TLC analyses were performed on 20 x 20 cm glass plates, using a 250 μ thickness of silicic

TABLE II

Recoveries of Isolated Sphingolipids

Preparation	Wet weight (WW) g	Total lipid (TL) % WW	Cerebrosides % TL	Sulfatides % TL	Sphingomyelins % TL
Normal corpus callosum	13.4	18.1	16.7	5.4	2.2[a]
MS white matter	4.5	7.9	18.9	6.9	10.7[b]

[a]After Unisil, followed by Alumina, column purification.
[b]Directly from Unisil column (see text).

acid (Silica Gel H acc. to Stahl, E. Merck A.G., Darmstadt, Germany). Separations were carried out using chloroform-methanol-water, 65:25:4 (22), and chloroform-methanol-methylamine (30% aq.), 65:25:8 (23). General sprays used for detection of spots were 0.2% Rhodamine 6 G in 95% ethanol and 50% aq. sulfuric acid. Selective sprays were as follows: molybdate for phospholipids (24), orcinol-sulfuric acid for glycolipids (25), ninhydrin for aminolipids (26) and benzidine-hypochlorite for sphingolipids (25).

Gas Liquid Chromatographic Analyses

GLC analyses were performed on a 6 ft by 1/8 in. glass column packed with diethylene glycol succinate (DEGS), as a 4% coating on Gas Chrom Q (pre-packed by Beckman Instruments, Inc., Fullerton, Calif.). A Beckman GC-5 dual column instrument was used, equipped with dual hydrogen flame detectors. Helium was used as a carrier gas, and analyses were performed at various temperatures between 120 and 160 C. Aldehydes were identified by use of various techniques and application of criteria described in an earlier publication (18). These are: retention time identity with standard aldehydes, retention time predictability from semilogarithmic carbon number plots, formation of bisulfite addition compounds, reduction by sodium borohydride and catalytic hydrogenation (for unsaturated aldehydes). Retention time data used in identification of sphingosine base-derived aldehydes are presented in Table I.

RESULTS AND DISCUSSION

For a number of reasons described below, the data obtained from these experiments has limited, yet real value, and must be evaluated with care. An unavoidable problem encountered in collecting data was that only one sample of MS tissue was available. A previous publication (18) has established that non-ganglioside polar lipid sphingosine base composition is quite uniform among a number of neurologically normal, adult human brains.

Recent data from this laboratory (34) show that this is also the case for individual, normal adult sphingolipids. Normal data reported here, while representing a

TABLE III

Sphingosine Base Composition of Normal and MS White Matter

Sphingosine bases	Sphingolipids					
	Cerebrosides		Sulfatides		Sphingomyelins[a]	
	Normal %	MS %	Normal %	MS %	Normal %	MS %
16:Dihydrosphingosine	1	.[b]	---	---	---	---
18:Dihydrosphingosine	4	2	2	5	4	3
18:Sphingosine[c]	95	96	98	93	95	94
(O-methyl component)	(18)	(21)	(21)	(19)	(12)	(14)
20:Sphingosine (?)[d]	---	---	---	---	1	3
Unidentified[e]	--	2	---	2	---	---

[a]Sphingomyelin data are reported with the reservation that in the normal case, sphingomyelin recovery was poor (see text).
[b]Aldehydes and suspected aldehydes which were present at <1% are not included.
[c]The data reported for 18:sphingosine include the 0-methyl component, the contribution of which is noted in parentheses.
[d]The lack of certainty of this identification is explained in the text.
[e]See note [c], Table I.

TABLE IV

Sphingosine Base Composition of Nonganglioside Polar Lipids of Myelin and Whole White Matter

Material analyzed		Sphingosine base[a]		
Preparation[b]	Amount, g	16:Dihydro-sphingosine, %	18:Dihydro-sphingosine, %	18:Sphingosine, %
Whole corpus callosum	15.3	1	4	95
Myelin, wet	8.4	1	4	95
Myelin, lyophilized	0.5	1	4	95

[a]Components which were present at <1% are not included.

[b]Each of these was prepared from the same corpus callosum white matter (neurologically normal, young adult male).

single corpus callosum, can therefore be considered representative of normal cases. Another problem is that of recovery of sphingolipids from the isolated process. Total recovery of lipids from Unisil columns ranged from 80% to 85% on a weight basis. Since silicic acid is known to degrade phosphatidyl ethanolamine and phosphatidyl serine (28,29), and there is no reason to suspect significant degradation or irreversible adsorption of neutral lipids under the conditions used, it is reasonable to assume that sphingolipid recoveries were in the order of 80% to 85%. Recovery data for both normal and MS sphingolipids are presented in Table II. Use of semi-quantitative TLC, whereby systematically diminishing aliquots of samples were subjected to TLC development followed by charring, established that recoveries of cerebrosides and sulfatides from Florisil columns were quite good; as an estimate, they were better than 80%. The same cannot be said for sphingomyelins.

In the case of sphingomyelins from normal white matter, loss in the order of 50% (in this case) was observed after Alumina chromatography. We do not think that the loss was selective in terms of molecular species, because in other sphingomyelin isolations, recoveries varied widely, while sphingosine base composition remained essentially constant. But, in order to report this work rigorously, the sphingomyelin data have been marked, (Table III) to indicate the possibility, albeit not probability, that the sphingomyelins analyzed were not representative of all the sphingomyelins originally present. It was fortunate that, in the case of MS sphingomyelins, the sphingomyelin fraction from the Unisil column was very clean, containing no other sphingolipids with the exception of traces of what appeared to be gangliosides. For this reason, MS sphingomyelins collected from the Unisil column were used directly for methanolysis. Purity of isolated sphingolipids was determined by analytical TLC, chromatographing heavy loads and using two different solvent systems, and examining plates by the various sprays previously listed. With the sole exception of possible ganglioside traces in MS sphingomyelin, all other fractions yielded a single spot under these conditions. Since only one normal and one MS brain sample were used to accumulate the data in Table II, and in view of the difficulties cited in recovering normal sphingomyelin, it would be presumptuous to draw general conclusions from these data.

The data in Table III reveal nearly no difference in sphingosine base composition between the same sphingolipid classes of normal and MS white matter. The only significant difference noted in these experiments is the presence, in MS cerebrosides and sulfatides, of an unidentified aldehyde at relative retention time of 5.00, presumably representing an unidentified base, which was not found in the corresponding normal sphingolipids. Since this was found at the 2% level, and only one sample of MS tissue was available for analysis, the meaning of this finding is not clear, and will have to await further investigation. A finding more likely to be significant is that both normal and MS sphingomyelins appear to contain small amounts of 20: sphingosine, which was not found in cerebrosides and sulfatides. Our reasons for refraining from claiming this as proved are the following: First, there was not enough material for a definitive catalytic hydrogenation study; and second, traces of what appeared to be gangliosides were found in purified MS sphingomyelins. Gangliosides would indeed contribute 20:sphingosine, but the trace quantities noted were considered insufficient to contribute even 1%, much less the 3% noted. Third, a problem arises from the

much better recovery of MS sphingomyelins than of normal sphingomyelins. A summary of these points is that the first reason leaves doubt as to the identity of the compound we believe to be 20:sphingosine, and the second and third reasons make quantitation of this compound quite impossible.

No serious difficulties were encountered in obtaining compositional data for cerebrosides, sulfatides or nonganglioside polar lipids, but when the reflux (2 N in HCl) methanolysis technique was applied to sphingomyelins, frustratingly variable results were obtained. Much more gratifying results were obtained by using Gaver and Sweeley's (27) sealed-tube (1 N in HCl) method for methanolysis of sphingomyelins. The data presented in Table II strengthen, in addition, these investigators' claim that their method produces lower quantities of the main side-product, 0-methyl 18:sphingosine. A recent report from Sweeley's laboratory (31) notes that the 0-methyl ether produced is predominantly the 5-0-methyl isomer. We were, in fact, unable to find significant quantities of the other expected 0-methyl ether, i.e., the aldehyde which might represent 3-0-methyl 18:sphingosine.

Since only 4.5 g of MS plaque-containing white matter were available for this study, we were forced to use whole white matter, rather than isolated myelin. In order to check the validity of using whole white matter data to represent myelin composition we had performed in earlier studies, sphingosine base analyses of nonganglioside polar lipids in ultracentrifugally isolated adult human myelin, and in corresponding white matter. Since myelin pellets prepared in this manner contain more water than does whole tissue, the possibility that the presence and amount of water present might influence the extraction of total lipids was considered and controlled by parallel analysis of a lyophilized myelin preparation. Polar lipids were prepared from a portion of whole corpus callosum white matter, from a different neurologically normal adult brain than that used for isolated sphingolipid studies. Other portions of this same material were used to isolate myelin, part of which was lyophilized. It is apparent, from the data in Table IV, that no differences were found among the three preparations analyzed. It remains entirely possible that some differences might be manifested if specific classes of sphingolipids were compared. A still better experiment will be the comparison of individual sphingolipids from MS and normal myelin when more MS tissue becomes available.

Finally it should be noted that Michalec and Holman (33) recently reported experiments in which acid-catalyzed methanolyses, including the two methods used by us, yielded incomplete liberation of sphingosine bases from sphingolipids. We have found the completeness of methanolysis to vary considerably, the variation apparently dependent on a number of factors. In most cases, completeness of methanolysis was in the order of 90%. Initial experiments failed to reveal significant selectivity in the variable release of sphingosine bases from sphingolipids (unpublished observations). We therefore consider our data to be valid in describing the chain length distribution of sphingosine bases in the sphingolipids investigated.

ACKNOWLEDGMENTS

Technical assistance by Miss Patricia Joy Chu and Mrs. Olivia Masih White. H. M. Zimmerman, Montefiore Hospital and Medical Center, provided the sample of MS brain. This investigation was supported in part by Grant 489-B2 from the National Multiple Sclerosis Society and by Grant NB-06770-03 from the National Institute of Neurological Diseases.

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[Received December 9, 1968]

Composition and Biosynthesis of the Major Hexose Containing Sphingolipids of Pig Leucocytes

GABRIEL M. LEVIS, Biochemical Research Laboratory, Department of Clinical Therapeutics, University of Athens, Alexandra Hospital, Athens, 611, Greece

ABSTRACT

The oligohexosyl ceramides of two preparations of blood leucocytes taken from large white pigs, weighing 80 kg were analyzed and found to comprise as major constituents trihexosyl ceramide, with glucose-galactose in a molar ratio of 1:2, and glucosyl ceramide. The mean value of the two preparations was 4.9% and 1.35% of the total lipids, respectively, for the two glycolipids. Lactosyl ceramide is the major component of human leucocytes, in contrast to the pig leucocytes where it was detected in minor quantities. U-^{14}C-glucose, incubated with bone marrow cells of young pigs (20 kg living weight), was mainly incorporated into the carbohydrate moiety of glucosyl ceramide and trihexosyl ceramide. Further analysis of this compound indicated that all three hexoses were labeled with ^{14}C and that the glucose attached to the ceramide had the highest specific activity. Time studies showed a decline in the incorporation of ^{14}C into glucosyl ceramide as compared to its incorporation into trihexosyl ceramide.

INTRODUCTION

Sphingolipids containing one to four hexoses are found in most of the non-nervous tissues with a variation in composition depending upon the species, and normally in low concentrations (1). Human leucocytes are an exception in this respect, since they contain high concentrations of dihexosyl ceramide (2). The biological functions of the neutral oligohexosyl ceramides are unknown, though it is interesting to note that they can function as haptens (3) and that they are exclusive in the plasma membrane (4).

Though the pathways of biosynthesis of the mono- and dihexosyl ceramides have been described (5-10), pathways of the higher member have not been elucidated to this date. Such studies, performed in various mammalian tissues, may furnish an explanation of the large diversity in total amount and composition which exists among various mammalian tissues and species.

As a continuation of our studies on the hexose-containing sphingolipids of leucocytes, the same kind of tissue was analyzed from large white pigs and trihexosyl ceramide was found at an increase concentration, thus differing substantially from human leucocytes in their composition. To explain these findings, the synthesis of glycolipids in pig bone marrow cells was attempted and results are presented, indicating that U-^{14}C-glucose is mainly incorporated into glucosyl ceramide and trihexosyl ceramides.

MATERIALS AND METHODS

Isolation of Leucocytes

Large white pigs (weighing about 80 kg) were shot with a slaughtering gun, and blood (9 vol) was collected by heart puncture and mixed with 1 vol of isotonic 5% EDTA solution of pH 7.5. The blood was then mixed with 0.20 vol of 10% polyvinylpyrolidone (2) and the supernatant, following sedimentation of the erythrocytes, was centrifuged for 15 min at 50 x *g*. The red cells contaminating the sedimented leucocytes were hemolyzed by exposure to hypotonicity for 30 sec. Osmotic normality was reestablished with one third of the volume of 3.6% sodium chloride solution. The cell suspension was gently homogenized in a glass Potter-Elvehjem homogenizer with a loosely fitting Teflon pestle and centrifuged at 50 x *g* for 15 min. The supernatant, composed of the ghosts of the red cells, was discarded and the leucocytes rewashed with isotonic saline and centrifuged. All operations took place at about 10 C.

Preparation and Incubation of Bone Marrow Cells

Large white pigs, weighing about 20 kg, were used for the isolation of bone marrow cells. Following the killing of the animal, the femurs were removed, freed of tissue and placed in crushed ice. Immediately thereafter the ends of the femurs were cut off with a saw and the marrow extruded, by scraping the cavity, into cold 0.25 M sucrose solution of pH 7.4, containing heparin (50 mg/liter). The tissue was gently homogenized in plastic tubes with a loosely fitting plastic pestle, filtered through cheese cloth and centrifuged in the cold at 50 g for 15 min. The fat formed a pellicle floating at the surface of the sucrose solution and was removed manually. The cellular sediment was washed with the sucrose-heparin solution by repeated aspiration into a siliconized pasteur pipette with a narrow tip and the contaminat-

ing red cells were hemolyzed as described for the preparation of blood leucocytes, and suspended in enough 0.9% sodium chloride solution to render approximately 1.5 x 10^8 cells per 0.2 ml. Smears of the cells were prepared and the differential count of a typical preparation was: myeloblast, 0.7%; promyelocytes, 2.4%; myelocytes, 6.4%; metamyelocytes, 21.8%; polymorphonuclear, 54.2%; lymphocytes, 1.3%; plasma cells, 0.3%; monocytes, 0.4%; reticulum cells, 0.7%; pronormoblasts, 0.7%; and normoblasts, 11.1%. The percentage of the eosinophils in the myelocytes, metamyelocytes and polymorphonuclear was increased. Several siliconized McCartney flasks were prepared containing 0.2 ml of the cell suspension, 15 μc of U-^{14}C-glucose (sp. act. 10 μc/0.2 μmole) in 0.05 ml and 0.75 ml of a Ringer bicarbonate solution of pH 7.4 (11). The flasks were gased with O_2-CO_2 (95%:5%) and incubated at various time intervals at 37 C with shaking. The reaction was stopped by the addition of 0.1 ml of 10% citric acid.

Extraction of Total Lipids and Separation of Oligohexosyl Ceramides

Suspensions of leucocytes or bone marrow cells in sodium chloride, or the whole incubation mixture of bone marrow cells, were extracted according to Folch et al. (12), as modified by Miras et al. (2). When the extract originated from incubations with radioactive materials, it was subjected to four washings with the theoretical upper phase (12). Separation of the oligohexosyl ceramides was performed on a Florisil column (activated Magnesium Silicate, Sigma Chem. Co., St. Louis, Mo.) using 5 g for up to 10 mg of total lipids. The neutral lipids, cholesterol and ceramides, were eluted from the column with a mixture of chloroform-methanol (99:1 v/v). All the hexose-containing sphingolipids were obrained with chloroform-methanol (1:2 v/v). This fraction was purified as described previously (2) by mild alkaline hydrolysis and silicic acid chromatography. Separation into neutral and acidic fractions was performed on a DEAE cellulose column (13). To separate the total neutral oligohexosyl ceramide fraction into monohexosyl ceramides (CMH), dihexosyl ceramides (CDH) and trihexosyl ceramides (CTH), the material of this fraction was applied to Silica Gel H borate thin layer plates (14) and chromatographed with Chloroform-methanol-water-ammonia (100:45:4:0.5 v/v). When separation of CDH from CTH was incomplete, these compounds were once more separated on Silica Gel H plates with chloroform-methanol-water (65:25:4 v/v). Hexose-containing sphingolipids of known composition were simultaneously run as reference standards. The glycolipids were located and extracted from the plate as described by Svennerholm and Svennerholm (15), and the major glycolipid weighed. The purity of the isolated compounds was checked by thin layer chromatography with the same solvent system and the spots were developed by spraying the plates with sulfuric acid-water (1:1 v/v) and charing at 130 C for 15 min.

Methods of Analysis

Total hexose was assayed with the anthrone method in the hydrolysates of the hexose-containing sphingolipid, as previously described (2). Standards of glucose or mixtures of glucose-galactose (1:1) and (1:2) by weight, were used. Glucose was assayed enzymatically with glucose oxidase (16). Total nitrogen was determined by direct nesslerization of the digested lipids (17). Partial acid hydrolysis and paper chromatography of the hexose were performed as previously described (2). Infrared analysis was performed on KBr pellets with a Beckman IR-5 spectrophotometer. The Elson-Morgan reaction was performed as described elsewhere (18).

RESULTS

The total lipids extracted from two preparations of pig leucocytes were analyzed for oligohexosyl ceramides. Examination of the crude extract by thin layer chromatography revealed two diphenylamine-positive spots with mobilities similar to those of glucosyl ceramide and trihexosyl ceramide, which were isolated from pig spleen and characterized by analysis of products of partial and total acid hydrolysis. The composition of the isolated oligohexosyl ceramides is shown in Table I and compared to the composition of the corresponding fraction from human leucocytes, as previously reported (2). The chromatographic patterns of the ceramide oligohexosides from the leucocytes of the two species are shown in Figure 1. Characterization of glucosyl ceramide and lactosyl ceramide was based on their chromatographic properties and on the analysis of their carbohydrate residue after strong acid hydrolysis. Their quantification was based on total hexose determination, assuming a molecular weight of 620 for their lipid residue. The major neutral hexose-containing sphingolipid, as shown in Figure 1, moved at a considerably lower R_f than lactosyl ceramide. The infrared spectrum showed the following bands: strong doublets at 1650 cm^{-1} and 1550 cm^{-1}; strong band near 1490 cm^{-1}; weak bands at 1200 cm^{-1}, 980

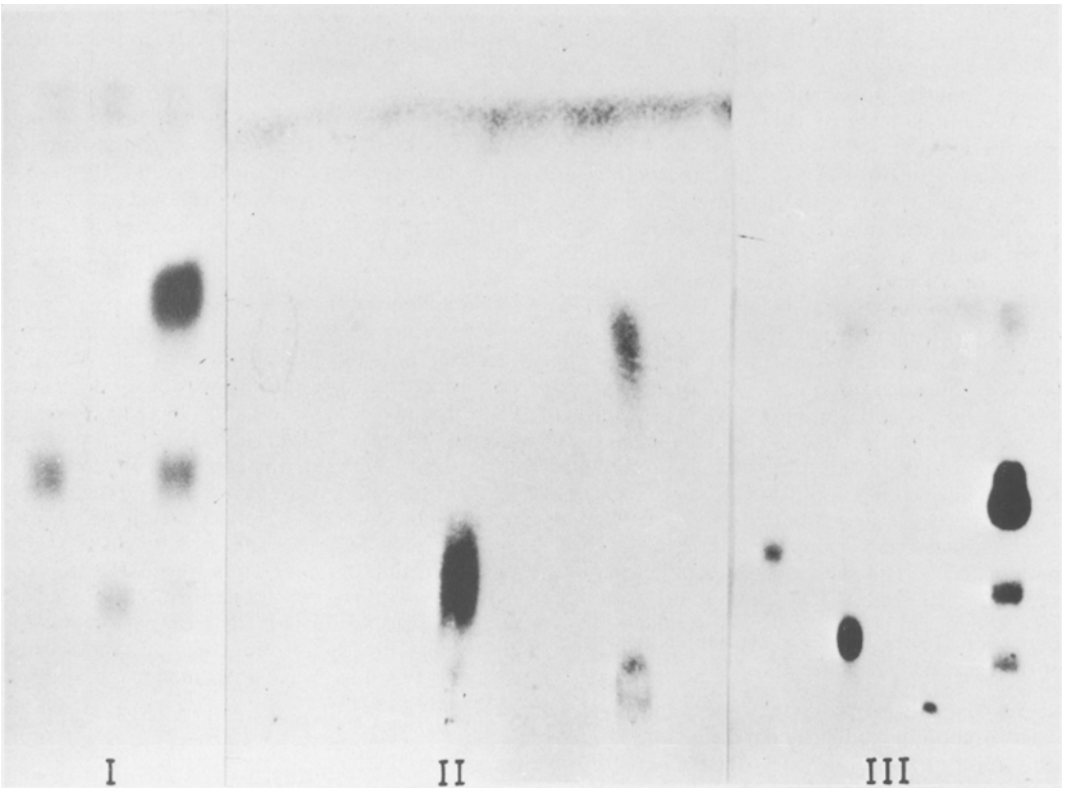

FIG. 1. Thin layer chromatograms of the hexose-containing sphingolipids of human and pig leucocytes. (I) Silica gel G plates (Merck, Darmstadt)chromatographed in chloroform-methanol-water (65:25:4 v/v). From left to right: (a) total glycolipids from human leucocytes; (b) trihexosyl ceramide and lactosyl ceramide from pig leucocytes; (c) mixture of reference standards from pig spleen, from the top glucosyl ceramide, lactosyl ceramide and trihexosyl ceramide. (II) Silica gel H borate plate, chromatographed in chloroform-methanol-water-ammonia (140:45:4:0.5 v/v). From left to right: (a) glucosyl ceramide from pig leucocytes; (b) galactosyl ceramide; (c) standard glucosyl cceramide. (III) Silica gel G F254 plate (Merck, Darmstadt), chromatographed in chloroform-methanol-water (65:25:4 v/v). From left to right: (a) lactosyl ceramide (lignoceric derivative, synthetic); (b) trihexosyl ceramide from pig leucocytes; (c) the acidic glycolipid of pig leucocytes; (d) mixture of reference standard from the top galactosyl ceramide, 3'-sulfato-galactosyl ceramide, sphingomyelin from brain.

cm^{-1}, near 815 cm^{-1} and 720 cm^{-1}; peaks relevant to sugar esterified sulfate groups at 1240 cm^{-1} (19) were not detected. The water soluble material obtained after strong acid hydrolysis indicated on paper chromatograms the presence of both glucose and galactose, the latter in excess. The mean values obtained from the material of the two preparations were: for total hexose, 36.5% (standard, glucose-galactose 1:2); for gluxose, 12.5%; the molar ratio of total hexose to total nitrogen was 2.81. The Elson-Morgan reaction was negative, thus ruling out the presence of hexosamine. Partial acid hydrolysis produced two less polar glycolipids, identified by hexose analysis and thin layer chromatography, as glucosyl ceramide and lactosyl ceramide. These data strongly suggest that the main hexose-containing sphingolipids of pig leucocytes are trihexosyl ceramide 5, containing glucose and galactose in a molar ratio of 1:2 with glucose attached to the ceramide. The material eluted in the acidic fraction of the DEAE cellulose column produced an infrared spectrum which showed a weak band at 1240 cm^{-1}, possibly indicating the presence of sugar esterified sulfate (19). On thin layer chromatography it moved at a lower R_f than 3'-sulfato-galactosyl ceramide (Fig. 1); this observation may suggest that this compound comprises more than one hexose in its molecule. Therefore, its quantification was based on total hexose determination with standard glucose-galactose (1:1) assuming a molecular weight of 620 for the lipid residue.

TABLE I

Oligohexosyl Ceramide Composition of Pig and Human Leucocytes

Compound	Per cent of total lipids Pig[a] 1	Pig[a] 2	Human[b]
Glucosyl ceramide	1.5	1.2	0.4
Dihexosyl ceramide	0.4	0.3	15.8
Trihexosyl ceramide	5.5	4.4	0.2
Glycolipid acidic	0.6	0.4	---
Total oligo-ceramides hexosyl	8.0	6.3	16.4

[a]Preparation 1, 175 mg of total lipids; preparation 2, 88 mg of total lipids.

[b]Data reported previously (2).

Incorporation of U-^{14}C-Glucose Into Oligohexosyl Ceramides

Bone marrow cells were incubated with U-^{14}C-glucose as described in the text. The isolation of the individual hexose-containing sphingolipids was achieved as described in the Methods section except that the radioactive spots were located with the aid of autoradiography. Results of time studies are shown in Table II. Duplicates containing 1.5 x 10^8 cells in 0.2 ml of NaCl (0.9%) and 15 μc/0.3 μmole U-^{14}C-glucose in 0.05 ml NaCl (0.9%) were incubated in a 0.75 ml Ringer-bicarbonate buffer. The total hexose containing sphingolipids were separated into sub-fractions by thin layer chromatography, located by autoradiography and extracted from the gel as described in the text. The results are the means of duplicates after subtraction of blank values derived from samples of silica gel of the same plate.

It can be seen that ^{14}C from U-^{14}C-glucose is mainly incorporated into glucosyl ceramide and trihexosyl ceramide and that, following a similar initial rate of incorporation into CMH and CTH, the incorporation into CMH shows a decline as compared to the incorporation into CTH. The intramolecular localization of radioactivity was examined in the 3 hr sample, after strong acid hydrolysis, and it was found that over 95% was associated with the carbohydrate residue.

The mode of biosynthesis of the carbohydrate chain of CTH was investigated further. For this purpose, bone marrow cells (5 x 10^9 cells) were incubated for 3 hr with U-^{14}C-glucose (750 μc/15 μmoles) in 50 ml of Ringer-bicarbonate medium, and the labeled CTH was isolated in a pure state as described in the text. We were thus able to isolate 3.5 mg of CTH with adequate specific activity for further analysis. Partial acid hydrolysis of this material

TABLE II

Incorporation of U-^{14}C-Glucose Into Glucosyl ceramide and Trihexosyl Ceramide by Pig Bone Marrow Cells

Time of incubation, min	Incorporation in cpm per 10^8 cells CMH[a]	CDH[a]	CTH[a]
15	330	0	400
60	770	40	1870
180	2150	260	4880

[a]CMH, monohexosyl ceramide; CDH, dihexosyl ceramide; and CTH, trihexosyl ceramide.

yielded radioactive dihexosyl ceramide and glucosyl ceramide which were separated and isolated by thin layer chromatography (Fig. 2). The specific radioactivities of these two degradation products and that of intact CTH were used for the calculations of the specific radioactivities of each one of the three CTH hexoses, the results are presented in Table III.

DISCUSSION

The results of the present study have shown that the composition of the oligohexosyl ceramides of pig and human leucocytes differ significantly in the hexose content of their major components, trihexosyl ceramide being the predominant glycolipid of pig leucocytes as compared to lactosyl ceramide, which, as reported previously, is the major glycolipid of human leucocytes (2). Nevertheless, the proportion of total oligohexosyl ceramide in the total lipid fraction of the leucocytes of both species,

TABLE III

Radioactivity of Trihexosyl Ceramide and Degradation Products

Compound	cpm per μmole of total hexose[a] x 10^3	cpm per μmole of each hexose[b] x 10^3
Trihexosyl ceramide third hexose	244	76
Dihexosyl ceramide[b] second hexose	168	63
Glucosyl ceramide[b] first hexose[c]	105	105

[a]Each glycolipid was subjected to strong acid hydrolysis and the water soluble fractions recovered from the reaction mixture were used for counting of radioactivity and total hexose determination.

[b]Degradation products of partial acid hydrolysis of the trihexosyl ceramide.

[c]First hexose attached to the ceramide.

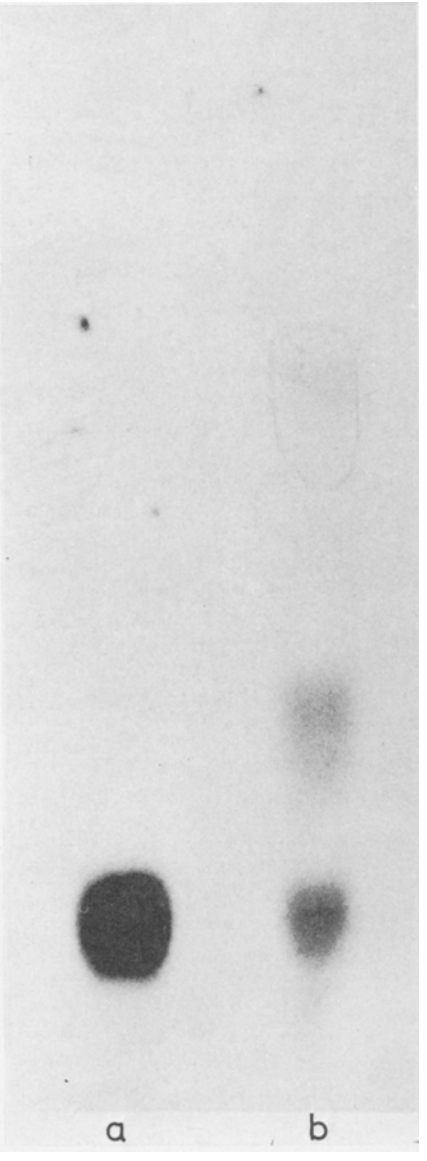

FIG. 2. Autoradiogram of a thin layer plate (Merck F254), chromatographed in chloroform-methanol-water (65:25:4 v/v). From left to right: (a) intact trihexosyl ceramide; (b) lipid fraction of products of mild acid hydrolysis, identified from the top as glucosyl ceramide, dihexosyl ceramide and trihexosyl ceramide.

especially in the latter, is unusually high for non-nervous tissues. Such observations confirm that a high concentration of these compounds is required for normal leucocyte function or structure, although their precise role still remains to be discovered. However, the possibility exists that these compounds may be related to the functions of bone marrow cells from which leucocytes are derived.

Since the leucocytes of both species do not contain glycolipids in sufficiently high quantities, which, by degradation could give rise to the accumulation of CDH or CTH, it was attempted to explain the differences in their glycolipid composition by examining the anabolic process. Specifically, the incorporation of U-^{14}C-glucose into the glycolipids of bone marrow cells, which are the site of production of the circulating leucocytes, was studied. These experiments have shown that U-^{14}C-glucose is incorporated mainly into glucosyl ceramide and trihexosyl ceramide. If it is assumed that the carbohydrate chain is built on the glucosyl ceramide, the low radioactivity found in the dihexosyl ceramide indicates either that this compound is not an intermediate in the biosynthesis of trihexosyl ceramide, or that it is quickly transformed to its higher member. Whatever the case may be, it can be predicted that the differences in glycolipid composition of human and pig leucocytes are due to differences in the mechanisms involved in the biosynthesis of these compounds in the bone marrow cells. It is worth mentioning here that during the present investigations lactosyl ceramide was not detected by thin layer chromatography in the glycolipid fraction of pig bone marrow cells.

The occurrence of radioactivity in all three hexoses of the CTH, obtained after incubation of pig bone marrow cells with U-^{14}C-glucose, is consistent with the de novo biosynthesis of the carbohydrate chain. The higher specific activity of the hexose attached to the ceramide, in conjunction with the decline in the incorporation of U-^{14}C-glucose into CMH as a function of the time of incubation, may suggest a precursor-product relationship between CMH and CTH. To our knowledge, this is the first report on the de novo biosynthesis of the carbohydrate chain of trihexosyl ceramide. Basu et al. (20) have reported the incorporation of radioactivity into lipids from UDP-N-acetyl-galactosamine in a brain particulate system, in the presence of dihexosyl ceramide. This may be considered as biosynthesis by the addition of a sugar to the carbohydrate chain of this glycolipid acceptor.

Trihexosyl ceramides have been reported to occur as normal constituents of the lipids of serum, liver, kidney and spleen of the various mammals (1). Therefore, the elucidation of the details of the enzymatic mechanism involved in their biosynthesis is not only important per se, but it is expected to provide answers to questions related to species and organ differences concerning their hexose containing sphingolipid composition. Other phenomena related to the

metabolism of trihexosyl ceramide are the accumulation of this compound in the kidneys of patients with Fabry's disease (21) and their implication in the changes produced by ascites tumors in the glycolipid composition of the kidneys of mice (22).

ACKNOWLEDGMENT

This work was supported in part by NIH Grant HE 10421.

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[Received December 24, 1968]

Specific Distribution of Fatty Acids in the Milk Fat Triglycerides of Goat and Sheep[1]

L. MARAI, W. C. BRECKENRIDGE and **A. KUKSIS**, Banting and Best Department of Medical Research, and Department of Biochemistry, University of Toronto, Toronto, Canada

ABSTRACT

The triglycerides of the fat globules of sheep and goat milk were isolated and separated into short and long chain lengths by silicic acid column chromatography. The short chain lengths comprised major triglycerides with 34-44 acyl carbon atoms and accounted for nearly 50% of the total milk fat. The long chain lengths contained major triglycerides with 40-54 acyl carbons. Stereospecific analyses of the short chain triglyceride fraction showed that of the 20-23 moles per cent of C_4-C_8 fatty acids present, at least 95% were specifically attached to the glycerol molecule in the position corresponding to carbon 3 of *sn*-glycerol. The distribution of the other fatty acids (C_{10} or greater) did not show such marked specificity for either the 1 or the 2 position. Although individual triglycerides were not identified, the specific placement of the fatty acids could best be accounted for by assuming a common pool of long chain 1,2-diglycerides which served as precursors of the bulk of both short and long chain triglycerides during milk fat synthesis.

INTRODUCTION

Stereospecific analyses of bovine milk fat have shown (1,2) that the butyric acid and other short chain fatty acids, which are specifically derived by de novo synthesis in the mammary gland (3), are largely or exclusively esterified to the 3 position of the glycerol molecule, *sn*-nomenclature used throughout (4). The distributions of the other fatty acids (C_{10} or greater), which are derived from diet and the depot fat, did not exhibit such marked specificity for either the 1 or the 2 position. These data support the hypothesis (5) that the short chain fatty acids are esterified with long chain diglycerides, or are substituted in glycerophosphatide intermediates, in the final step of milk fat biosynthesis. The milk fats of sheep and goat are also rich in butyric and other short-chain acids (6), but their intraglyceride distribution has not been ascertained. A demonstration of comparable stereospecificity in these species would establish the generality of the phenomenon in the ruminants, and might permit extrapolation of data to nonruminant species which also derive their milk fats by mobilization and partial de novo synthesis of fatty acids in the mammary gland.

MATERIALS AND METHODS

The chemical reagents, solvents, chromatographic materials and analytical standards were as described (2). Fresh samples of raw sheep and goat milk were obtained from local farms. The goat milk (450 ml) was collected from one animal, while the sheep milk (360 ml) was pooled from three ewes. The triglycerides were isolated from the milk fat globules by extraction with chloroform-methanol (2:1) as described for cow's milk (7). The various triglyceride preparations were free of contamination with free fatty acids, diglycerides, and any other common lipids by chromatography on columns or thin layers of silicic acid, as described below.

Separation of Short and Long Chain Lengths

Triglycerides of short and long chain length were resolved by chromatography on columns of silicic acid essentially as described by Blank and Privett (8). The fractions obtained from 1.2 g of total milk fat were pooled in two nearly equal portions; the least polar one (0.6-0.7 g) provided the long chain length, the more polar one (0.5-0.6 g) gave the short and medium chain length triglycerides. The latter fraction comprised major triglycerides with 32-46 acyl carbon atoms, the former long chain triglycerides with 40-54 acyl carbons. Smaller quantities of short and long chain triglycerides were resolved by thin layer chromatography (TLC) as previously described (9). This method was also used to resolve the short and long chain diglycerides released from the short chain triglyceride fraction by hydrolysis with pancreatic lipase.

Stereospecific Analysis

The positional distribution of the fatty acids in the short and long chain triglycerides was determined by the method of Brockerhoff as previously described (2). After a brief hydrolysis with pancreatic lipase, the reaction mixture

[1]Presented in part at the AOCS Meeting, New York, October 1968.

TABLE I

Molecular Weight Distribution of Short and Long Chain Triglycerides of Goat and Sheep Milk Fat[a,b]

Carbon No.	Goat					Sheep				
		Short chain		Long chain			Short chain		Long chain	
	Original	Total	Residual[c]	Total	Residual	Original	Total	Residual	Total	Residual
24						Trace	0.2	0.2		
26	0.1	Trace	Trace			0.5	0.5	0.6		
28	0.3	0.2	0.1			0.8	1.3	1.2		
29	Trace					Trace	0.1	0.1		
30	0.7	0.5	0.7			1.2	2.2	2.2	0.1	0.1
31	0.1					Trace	0.2	0.1		
32	1.5	2.0	2.0			2.0	3.9	3.7	0.3	0.3
33	0.1	0.1	0.2			0.2	0.4	0.4		
34	3.9	5.9	6.1			4.0	7.7	7.5	1.0	0.9
35	0.5	1.0	0.7			0.7	1.2	1.1		
36	8.5	16.9	16.3			7.2	15.2	14.6	2.3	2.2
37	0.7	1.2	0.7			1.3	1.9	2.0		
38	12.4	26.9	25.2	Trace	Trace	11.4	23.9	23.6	4.4	4.3
39	Trace			Trace	Trace	0.7				
40	10.4	21.8	21.5	1.0	1.1	11.3	20.3	20.0	5.5	5.5
42	8.1	10.5	11.3	4.5	4.6	6.4	7.2	7.4	5.9	6.0
43	Trace			0.3	0.2					
44	7.4	5.4	6.1	7.5	7.6	5.4	3.7	4.0	6.5	6.6
46	5.9	2.7	3.2	8.3	8.4	5.5	2.4	2.7	7.3	7.5
48	7.6	1.0	1.5	13.4	13.5	7.2	1.8	2.0	10.8	10.6
50	12.3	1.4	1.8	25.1	24.6	10.5	2.2	2.4	16.3	16.4
52	13.1	1.2	1.7	27.3	27.4	13.5	2.1	2.4	22.2	22.3
54	6.2	1.3	0.9	12.1	12.2	9.5	1.5	1.7	15.4	15.6
56	0.2			0.2	0.1	0.4	0.1	0.1	1.0	0.8
58						0.3			0.6	0.6
60						Trace			0.4	0.3

[a]Short and long chain lengths resolved by chromatography on silicic acid columns.

[b]Values are given in mole percentage.

[c]Triglycerides recovered following partial hydrolysis with pancreatic lipase.

was extracted with diethyl ether without acidification. The ether extracts were concentrated and the mono-, di- and triglycerides isolated by TLC. Portions of the mono- and diglycerides were then acetylated by reaction with acetic anhydride-pyridine. The acetates were examined by gas chromatography along with the unhydrolyzed triglycerides and the yield and composition of the products of the lipase hydrolysis assessed by reference to tridecanoin which was added as internal standard. Other aliquots of these fractions were transbutylated and the fatty acid composition determined by gas liquid chromatography (GLC).

The rest of the diglycerides was converted into glycerophosphatidyl phenols by treatment with phenyl dichlorophosphate and the reaction products isolated by TLC. The digestion with phospholipase A was performed as described by Brockerhoff (10), except that less phosphatidyl phenol (50 mg) and only one half as concentrated a buffer was used. The phosphatides derived from 1,2-diglycerides yielded the 2-fatty acid, while those from 2,3-diglycerides having the L-configuration, were not attacked. After incubation for 4 hr at room temperature, the reaction products were isolated by TLC (2).

Gas Liquid Chromatography

GLC analyses of fatty acid butyl esters, monoglyceride diacetates, diglyceride monoacetates and di- and triglycerides were done as previously described (2).

RESULTS AND DISCUSSION

Starting Materials

The overall fatty acid and triglyceride distributions of the goat and sheep milk samples were similar to those described earlier (6). Table I gives the composition of the triglycerides in the short and long chain fractions and compares them to those of the total from which they were derived by chromatography on silicic acid. While the short chain triglycerides (26-42 acyl carbons) have been nearly completely resolved from the long chain length

TABLE II

Fatty Acid Composition of Short and Long Chain Triglycerides of Goat and Sheep Milk Fat[a,b]

Fatty acids	Goat					Sheep				
		Short chain		Long chain			Short chain		Long chain	
	Original	Total	Residual[c]	Total	Residual	Original	Total	Residual	Total	Residual
4:0	5.1	10.2	10.3			4.4	8.0	7.8		
6:0	4.4	8.2	8.5			3.6	7.2	7.2	0.5	0.6
8:0	2.6	4.7	4.8	0.9	0.5	2.4	3.8	3.6	1.3	1.1
10:0	7.8	8.8	9.0	6.4	6.3	5.5	7.5	7.1	4.0	4.6
10:1	0.3	0.3	0.3			0.2	0.3	0.3	0.1	0.1
12:0	3.8	3.9	3.8	2.7	2.3	3.5	4.3	3.9	2.8	2.6
12:1						0.1	Trace	Trace	0.1	Trace
14:0	9.6	10.3	10.0	10.5	9.4	9.8	11.1	10.5	9.6	9.6
14:1	0.2	0.5	0.4	0.5	0.5	0.6	0.5	0.5	0.8	0.9
15:0	2.0	1.5	1.7	2.5	2.3	2.8	2.2	2.7	3.1	2.7
16:0	26.0	24.6	24.9	28.7	28.6	21.2	21.2	21.3	21.5	22.2
16:1	1.8	2.0	1.6	2.3	2.6	1.7	1.4	2.1	2.3	2.2
17:0	0.8	0.7	0.5	1.3	1.4	1.0	0.8	0.6	1.1	1.1
18:0	9.9	6.0	6.6	12.7	13.6	14.0	9.8	10.6	16.8	16.9
18:1	20.6	14.0	14.0	26.4	26.9	21.8	15.1	15.0	28.4	28.5
18:2	2.7	2.0	1.8	3.1	3.7	4.4	3.7	3.4	4.6	3.7
18:3						2.6	2.5	2.7	3.0	2.7
20:1	2.4	2.0	1.8	1.8	1.9	0.4	0.5	0.5	Trace	0.5
20:2	Trace	0.3	Trace	0.2	Trace	Trace	0.1	0.2	Trace	Trace

[a]Short and long chain lengths resolved by chromatography on silicic acid columns.

[b]Values are given in mole percentage.

[c]Triglycerides recovered following partial hydrolysis with pancreatic lipase.

TABLE III

Molecular Weight Distribution and Fatty Acid Composition of the Diglycerides Derived From Short Chain Triglycerides by Lipase Hydrolysis[a,b]

Carbon No.	Diglyceride acetates				Carbon No.	Fatty acids			
	Goat		Sheep			Goat		Sheep	
	Original	Residual[c]	Original	Residual		Original	Residual	Original	Residual
16	0.7	2.1	1.3	3.8	4:0	8.5	15.0	9.5	11.7
18	1.4	3.4	2.6	6.3	6:0	6.9	12.1	6.8	10.6
20	7.9	14.6	7.4	15.4	8:0	4.0	5.7	2.6	5.1
21		1.0	1.7	3.4	10:0	9.6	10.6	6.0	10.0
22	20.8	36.0	14.0	25.6	10:1	0.3	0.4	0.6	0.4
23			0.8	1.3	12:0	4.0	2.8	4.0	4.6
24	21.8	33.2	24.0	40.1	12:1				
25					14:0	13.0	10.7	12.9	10.8
26	7.9	7.8	10.9	3.5	14:1	0.8	1.1	0.8	0.9
27					15:0	1.0	1.5	2.6	2.3
28	5.0	1.5	3.9	0.6	16:0	26.0	16.4	22.0	12.6
29					16:1	2.3	1.2	1.2	1.2
30	4.0	0.4	4.3		17:0	0.7	0.6	0.6	0.8
31					18:0	5.1	3.1	8.8	7.1
32	5.6		4.8		18:1	13.6	13.9	16.3	15.0
33			0.3		18:2	2.0	2.5	3.3	4.3
34	9.9		8.0		18:3			1.6	2.0
35					20:1	1.9	2.1	0.4	0.4
36	10.1		9.1		20:2	0.3	0.3		0.2
37									
38	4.1		6.4						
40	0.8		0.4						

[a]Mixed 1,2- and 2,3-diglycerides released by partial hydrolysis with pancreatic lipase.

[b]Values are given in mole percentage.

[c]2,3-Diglycerides recovered from TLC of mixed 1,2- and 2,3-diglycerides.

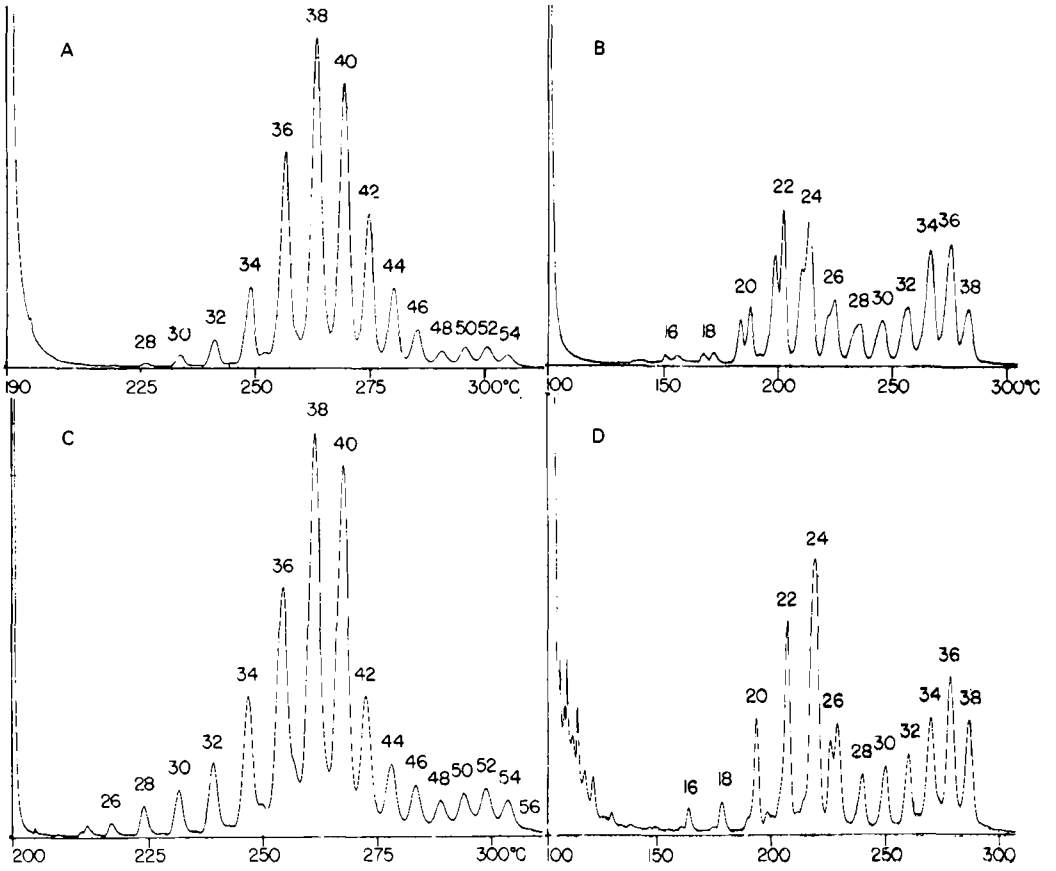

FIG. 1. GLC separation of short chain triglycerides and the derived diglyceride acetates. The peaks are identified by the total number of acyl carbon atoms. Beckman GC-4 gas chromatograph; columns 3% (w/w) JXR (dimethylpoly-siloxane gum) on Gas Chrom Q (100-120 mesh); temperature program as shown. A, short chain triglycerides of goat milk fat; B, diglyceride acetates of A; C, short chain triglycerides of sheep milk fat; D, diglyceride acetates of C.

glycerides (44-54 acyl carbons) of the goat milk, the removal has been less effective for the sheep milk glycerides. Furthermore, in both cases the short chain fraction still contains significant amount of long chain triglycerides (C_{44}-C_{54}). This contamination, however, was not sufficient to impare the reliability of the subsequent stereospecific analysis. The short chain fractions of the goat and sheep milk triglycerides made up 52 and 40 moles per cent respectively, of the total milk fat triglycerides. A proportional summation of the distributions of the short and long chain glycerides gave the reconstituted total distributions which differed little from those of the corresponding original milk fats.

Pancreatic Lipase Hydrolysis

Table I also gives the composition of the triglycerides remaining after the limited exposure of the short and long chain triglycerides to pancreatic lipase. On the basis of the molecular weight distribution there is little indication of a selective hydrolysis of any triglyceride types. This is confirmed by the fatty acid composition given in Table II, which shows close qualitative and quantitative correspondence between the original and the residual glyceride mixtures.

The diglycerides from the short chain fraction were isolated in 15-20% yield. The distribution of their molecular weights and fatty acids is given in Table III. Since the C_4-C_8 acids comprised 20-23 moles per cent of the total fatty acids, almost all of the diglycerides of carbon number 24 or less (about 50% of total) must consist of one short and one long chain acid. The simultaneous appearance in the enzyme digest of short chain acids and diglycerides containing the same short chain acids was a further indication that the pancreatic lipase did

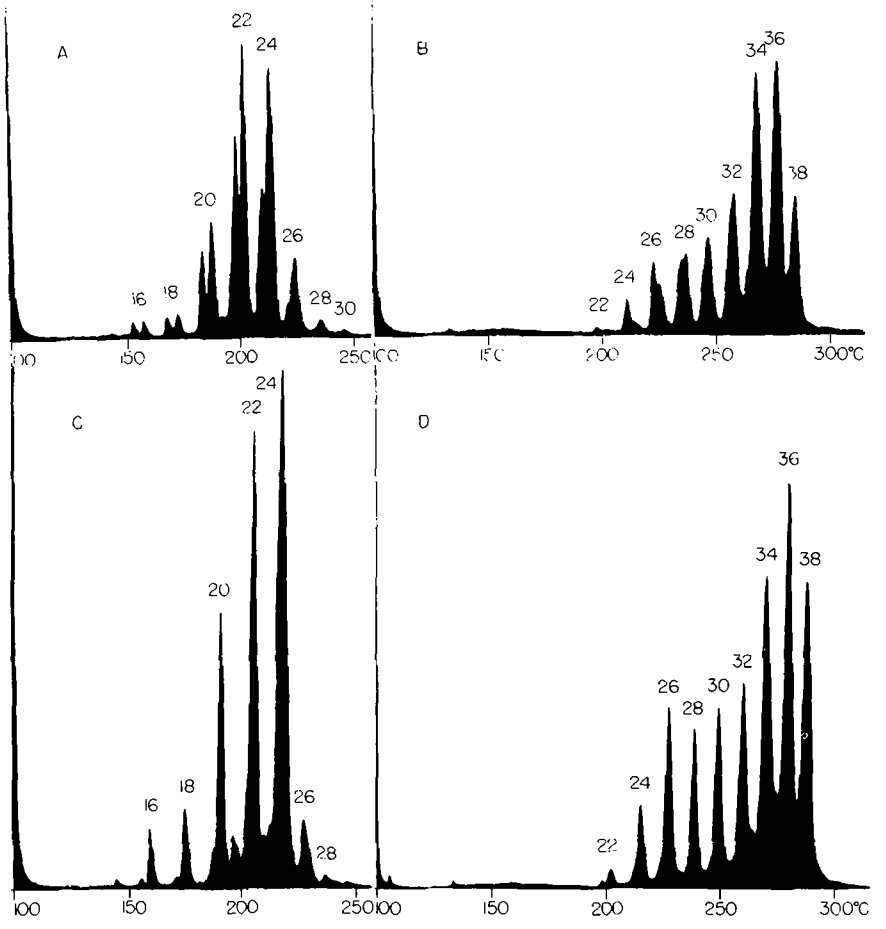

FIG. 2. GLC separation of the short and long chain diglyceride acetates of short chain triglycerides. Peak identification and instrumentation as in Figure 1. A and B, respectively, short and long chain diglyceride acetates of goat milk fat; C and D, respectively, short and long chain diglyceride acetates of sheep milk fat.

not discriminate between short and long chain fatty acids in the 1 and 3 positions of the short chain triglycerides. Figure 1 shows the GLC elution patterns of the original short chain triglycerides and of the diglycerides derived from them by pancreatic lipase hydrolysis. The diglyceride mixture shows the characteristic two hump distribution seen for the original milk fat of these species. These diglycerides can be resolved on the basis of chain length by means of TLC on silica gel. Figure 2 shows the GLC elution patterns recorded for the two diglyceride subgroups of the short chain triglycerides of goat and sheep milk fat. The molecular weight and fatty acid distribution of the short chain diglycerides approximated closely the composition of the residual lysophosphatidyl phenols discussed below. The long chain length diglycerides resembled those of the phosphatidyl phenols which were attacked by phospholipase A. These observations suggested that the TLC resolution of the diglycerides effectively divided them into the 1,2- and the 2,3-diglyceride types. Only the 2,3-diglycerides contained the short chain fatty acids.

Table IV gives the molecular weight and fatty acid distributions of the total diglycerides derived from the long chain triglycerides and of the residual phosphatidyl phenols (2,3-diglycerides) recovered following degradation of the mixed phosphatidyl phenols with phospholipase A. In both the goat and the sheep the glycerides have comparable distributions of molecular weights which are due to similarities in composition and distribution of the fatty acids. Furthermore, pancreatic lipase appears to

TABLE IV

Molecular Weight Distribution and Fatty Acid Composition of the Diglycerides Derived From Long Chain Triglycerides of Goat and Sheep Milk[ab]

Carbon No.	Diglyceride acetates				Carbon No.	Fatty acids			
	Goat		Sheep			Goat		Sheep	
	Original	Residual[c]	Original	Residual		Original	Residual	Original	Residual
20			1.2						
22			3.1						
24	0.2		5.9		4:0				
26	2.4		4.5		6:0			0.3	0.6
28	5.1	Not	4.6	Not	8:0	0.4	0.7	1.1	1.6
29			0.2		10:0	4.7	8.7	4.1	5.9
30	6.6	determined	6.5	determined	10:1				0.1
31			0.2		12:0	2.5	3.0	2.9	3.7
32	11.1		10.3		12:1			0.3	0.2
33			0.5		14:0	13.6	12.2	10.5	12.0
34	27.2		19.1		14:1	0.4	0.7	0.7	1.1
35					15:0	2.6	2.6	3.3	3.2
36	31.4		23.7		16:0	34.1	20.4	25.1	13.6
37					16:1	2.0	2.3	2.1	2.4
38	15.0		19.7		17:0	1.0	1.6	1.3	0.8
39					18:0	12.0	10.0	18.8	14.1
40	1.0		0.4		18:1	23.3	30.5	24.0	30.7
42					18:2	1.6	4.3	3.6	5.8
44					18:3			1.4	4.0
46					20:1	1.6	2.3	0.5	0.2
48					20:2	0.2	0.7	Trace	Trace

[a]Mixed 1,2- and 2,3-diglycerides released by partial hydrolysis with pancreatic lipase.

[b]Values are given in mole percentage.

[c]Residual phosphatidyl phenols containing 2,3-diglycerides.

have released nearly the same fatty acids from the long chain glycerides of both species, which would indicate that the two milk fats also possess comparable positional placement of the acids in these glycerides.

Positional Distribution of Fatty Acids

Table V presents the fatty acid compositions of the individual positions in the glyceride molecule of the short chain triglycerides. The fatty acids in position 1 were derived from the data for the lysophosphatidyl phenols. No C_4-C_6 acids were found in this location. The fatty acids in position 2 were determined by analysis of the monoglycerides liberated by pancreatic lipase and by analysis of the free fatty acids released by phospholipase A. Although the two determinations showed slight differences in the proportions of some of the medium chain length fatty acids, there were no signs of any short chain fatty acids. Furthermore, acetylation of the 2-monoglycerides, followed by GLC, gave molecular weight distributions which agreed closely with the fatty acid proportions, once again with no indication of any butyric or caproic acid residues in the 2 position.

The fatty acids in position 3 were obtained by summation and by subtraction as described in the footnotes to Table V. The two methods gave similar estimates for the major acids but the relatively large error (4-7%) precluded quantitative conclusions about the minor components. The C_4-C_{10} acids comprised approximately 75% and 60% of the total acids in the short chain triglycerides of goat and sheep milk, respectively. The remainder consisted of a variety of medium and long chain fatty acids which were due to the contamination of the short chain fraction by long chain triglycerides during the silicic acid column chromatography. The proportion of the long chain fatty acids in this fraction was consistent with the finding of only 20-23 moles per cent of short chain fatty acids in the original short chain triglyceride fraction. This distribution was also in agreement with the fatty acid composition of the 1,2- and 2,3-diglycerides recovered from the TLC of the total diglyceride fraction.

Table VI gives the fatty acid composition of the three positions of the glycerol molecule for the long chain triglycerides of goat and sheep milk. The most obvious characteristic of the acid distribution is the preferential association of myristic acid with the 2 position. Palmitic acid was distributed nearly equally between the 1 and 2 positions in the goat milk fat, while in

TABLE V

Positional Distribution of Fatty Acids in Short Chain Triglycerides of Goat and Sheep Milk Fat[a,b]

	Goat					Sheep				
	One	Two		Three		One	Two		Three	
Fatty acids	LPP	MG	FFA	Method A[c]	Method B[d]	LPP	MG	FFA	Method A	Method B
4:0				30.6	30.0				24.0	23.4
6:0				24.6	24.2				21.6	21.2
8:0	2.3	2.4	2.0	9.4	9.0	0.9	2.7	2.2	7.8	7.5
10:0	5.0	11.1	10.0	10.3	10.1	2.3	8.2	7.5	12.0	11.8
10:1		0.3	0.3	0.6	0.5		0.2		0.7	0.6
12:0	6.2	5.7	5.7	0.2	0.1	3.3	6.6	6.0	3.0	2.6
14:0	9.5	21.4	22.4	0.0	0.0	11.4	19.2	18.3	2.7	2.4
14:1	Trace	0.8	0.8	0.7	1.4		1.1	1.0	0.4	0.7
15:0	1.5	2.8	3.2	0.2	0.2	2.2	2.7	3.5	1.7	1.9
16:0	41.3	29.2	29.8	3.3	3.6	38.4	21.8	20.3	3.4	3.4
16:1	4.0	2.0	1.9	0.0	0.4	2.1	1.9	2.4	0.2	0.5
17:0	1.6	0.5	0.3	0.0	0.7	1.4	0.6	0.6	0.4	1.0
18:0	11.9	5.2	5.8	0.9	1.0	14.8	10.7	11.5	3.9	3.5
18:1	14.3	14.3	14.1	13.4	13.5	16.4	17.3	17.5	11.6	12.7
18:2	0.8	2.6	2.7	2.6	2.4	2.9	4.5	5.3	3.7	4.1
18:3						3.9	2.0	3.8	1.6	2.0
20:1	1.2	1.4	1.0	3.4	2.8	Trace	0.5	0.3	1.0	0.3
20:2	0.4	0.3		0.2	0.3		Trace		0.3	0.4

[a]Position relative to *sn*-glycerol 3-phosphate; LPP, L-lysophosphatidyl phenol produced from L-phosphatidyl phenols by phospholipase A; MG, 2-monoglycerides produced from original triglycerides by pancreatic lipase; FFA, free fatty acids produced from the L-phosphatidyl phenols by phospholipase A.

[b]Values are given in mole percentage.

[c]Values obtained by subtracting the sum of the acids of monoglycerides and lysophosphatidyl phenols from the original fatty acids.

[d]Values obtained by subtracting the monoglyceride acids from the acids of residual phosphatidyl phenols.

the sheep milk this acid was preferentially placed in the 1 position. Neither milk contained much palmitic acid in the 3 position, which in both species was occupied to a large extent by oleic acid. In both fats the stearic acid was preferred for the 1 position. These distributions are nearly identical to those reported earlier (10) for the long chain glycerides of cow's milk fat, except for oleic acid, which in the bovine milk samples was found in a somewhat higher concentration in position 1 than in the other two positions.

Table VII gives the fatty acid composition of the 1,2-diglycerides of the short and long chain triglycerides of goat and sheep milk. Although not matching in numerical values, the proportions of the acids in the corresponding diglyceride moieties are closely similar. These findings coincide with those made previously regarding the fatty acid composition of the 1,2-diglyceride moieties of the short and long chain triglycerides of bovine milk fat. As in the case of the bovine milk fats (11), it may be suggested that the 1,2-diglycerides are derived from a common pool during the resynthesis of the plasma triglycerides in the mammary gland. The peculiar milk fat triglyceride pattern could then be produced by a nearly random association of these diglycerides with the remaining C_4-C_{18} fatty acids.

The characteristic distribution of the fatty acids in the 1,2-diglycerides of the milk fats could arise either from any mono- and diglycerides derived by partial hydrolysis of blood triglycerides. The latter possibility, however, has been ruled out in the goat and cow by the results of pancreatic lipase hydrolyses of the milk fat and the blood triglycerides (12,13).

Mechanism of Biosynthesis

The extensive analyses of the milk triglycerides presented here and in previous publications (2,9,11) have allowed the exclusion of complete randomization or any simple modification of it as a system for milk fat triglyceride biosynthesis. The recognition of a common pool of 1,2-diglycerides in both short and long chain triglycerides of the ruminant milks is in agreement with the results obtained by Kinsella and

TABLE VI

Positional Distribution of Fatty Acids in Long Chain Triglycerides of Goat and Sheep Milk Fat[a,b]

	Goat					Sheep				
	One	Two		Three		One	Two		Three	
Fatty acids	LPP	FFA	MG	Method A[c]	Method B[d]	LPP	FFA	MG	Method A	Method B
4:0										
6:0									1.5	1.2
8:0	1.2	0.2	0.3	1.2	1.1	0.5	1.0	1.4	2.0	1.8
10:0	2.0	2.5	3.6	13.6	13.8	0.7	2.6	2.7	8.6	9.1
10:1		Trace	Trace				Trace	Trace	0.3	0.2
12:0	2.2	2.9	3.8	2.1	2.2	1.2	3.4	3.1	4.1	4.3
14:0	7.5	18.9	19.5	4.5	4.9	5.5	15.4	16.3	7.0	7.7
14:1		0.9	0.8	0.7	0.6	0.4	0.6	0.7	1.3	1.5
15:0	2.2	2.7	2.7	2.6	2.5	3.0	4.3	5.3	1.0	1.1
16:0	45.4	38.4	37.6	3.1	3.2	37.7	26.0	25.5	1.3	1.7
16:1	2.1	2.7	2.0	2.8	2.6	2.2	2.0	2.4	2.3	2.4
17:0	1.0	1.0	0.5	2.4	2.7	1.9	1.2	1.1	0.3	0.5
18:0	17.9	8.0	7.1	13.1	12.9	22.8	14.4	14.3	13.3	13.9
18:1	17.5	17.6	17.6	44.1	43.4	20.7	21.4	21.8	42.7	39.6
18:2	Trace	2.3	2.5	6.8	6.1	2.6	3.2	3.9	7.3	7.7
18:3						0.8	2.7	1.5	6.7	6.5
20:1	0.6	1.7	2.0	2.8	2.6	Trace	0.3	Trace	Trace	0.4
20:2	0.4	0.2	Trace	0.2	1.4	Trace	1.2	Trace	Trace	Trace

[a]Position relative to *sn*-glycerol 3-phosphate; LPP, L-lysophosphatidyl phenol produced from L-phosphatidyl phenols by phospholipase A; FFA, free fatty acids produced from the L-phosphatidyl phenols by phospholipase A; MG, 2-monoglycerides produced from the original triglycerides by pancreatic lipase.

[b]Values are given in mole percentage.

[c]Values obtained by subtracting the sum of the acids of monoglycerides and lysophosphatidyl phenols from the original fatty acids.

[d]Values obtained by subtracting the monoglyceride acids from the acids of residual phosphatidyl phenols.

TABLE VII

Distribution of Major Fatty Acids in the 1,2-Diglyceride Moieties of the Short and Long Chain Triglycerides of Goat and Sheep Milk[a,b]

	Goat				Sheep			
	Short chain		Long chain		Short chain[d]		Long chain	
Fatty acids	Pos. 1	Pos. 2[c]	Pos. 1	Pos. 2	Pos. 1	Pos. 2	Pos. 1	Pos. 2
8:0	2.3	2.2	1.2	0.3	0.9	2.4	0.5	1.2
10:0	5.0	10.5	2.0	3.0	2.3	7.8	0.7	2.6
12:0	6.2	5.6	2.2	3.4	3.3	6.3	1.2	3.2
14:0	9.5	22.0	7.5	19.2	11.4	18.8	5.5	15.8
16:0	41.3	29.5	45.4	38.0	38.4	21.0	37.7	25.8
16:1	4.0	2.0	2.1	2.4	2.1	2.2	2.1	2.2
17:0	1.6	0.4	1.0	0.8	1.4	0.6	1.9	1.1
18:0	11.9	5.5	17.9	7.5	14.8	11.0	22.8	14.3
18:1	14.3	14.2	17.5	17.6	16.4	17.4	20.7	21.6
18:2	0.8	2.6	Trace	2.4	2.9	5.0	2.6	3.5
20:1	1.2	1.2	0.6	1.8	Trace	0.4	Trace	0.2
20:2	0.4	0.3	0.4	0.1		0.1		0.6

[a]Position relative to *sn*-glycerol 3-phosphate.

[b]Values are given in mole percentage.

[c]Average values computed from independent estimates of the fatty acid composition of the monoglycerides and free fatty acids as explained in Table VI.

[d]The short chain triglycerides of sheep milk contained 3.9 and 3.0 moles per cent of 18:3 in positions 1 and 2, respectively.

McCarthy (14-16) with dispersed bovine mammary cells. Using 2-C^{14}-acetate and 3-C^{14}-glycerol, these workers observed specific activity-time curves which suggested that the milk fat triglycerides were produced by the acylation of 1,2-diglycerides with endogenously synthesized fatty acids.

The mechanism of biosynthesis of the 1,2-diglycerides is less obvious and possibly depends upon as yet unrecognized enzymic specificities. The present data reveal a continuous shift of fatty acids from position 3 to positions 2 and 1 as the chain length increases from C_6 to C_{14} and as the C_{18} acids become progressively more saturated. The extent to which this peculiarity may reflect the structural requirements of the triglyceride end products or the enzyme systems involved in their assembly may become more apparent when the molecular species of the milk fat glycerides are identified.

ACKNOWLEDGMENT

These studies were supported by grants from the Ontario Heart Foundation, and the Medical Research Council of Canada. The Medical Research Council also provided a Studentship to W. C. Breckenridge.

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[Received February 24, 1969]

A New Criterion in the Bioassay of Essential Fatty Acids: the Spontaneous Swelling of Rat Liver Mitochondria in Vitro

U. M. T. HOUTSMULLER, A. VAN DER BEEK and J. ZAALBERG,
Unilever Research Laboratory, Vlaardingen, The Netherlands

ABSTRACT

The spontaneous swelling in vitro of liver mitochondria from rats deficient in essential fatty acids (EFA) and normal rats is compared using the change in optical density as a criterion. Differences between both groups are observed only if high sucrose concentrations are used for the isolation of the mitochondria. The change in optical density can be described mathematically by the sum of two exponential functions. A correlation is found between the parameters of this function and the dose of sunflower seed oil fed to EFA-deficient rats, which makes the method useful as a criterion in an EFA bioassay. The EFA activities of several synthetic polyunsaturated fatty acids are compared using either growth rate or mitochondrial swelling as a criterion.

INTRODUCTION

The swelling in vitro of liver mitochondria from healthy rats has been studied fairly extensively under a great variety of conditions. On the other hand, swelling of liver mitochondria from rats deficient in essential fatty acids (EFA) has received only scant attention. Comparative studies by Hayashida and Portman (1) in 1960 and by Johnson (2) in 1963 did reveal a considerable increase in the rate of spontaneous swelling in vitro of EFA-deficient mitochondria, but the authors did not investigate whether the increased rate of swelling could be restored to normal by feeding EFA. It appeared useful to study this possibility, as the existence of a positive effect might make the swelling applicable as a criterion in a bioassay of essential fatty acids.

However, the procedures used for preparation of the mitochondrial suspensions by the above-mentioned authors were different. Johnson isolated the mitochondria in 0.30 M sucrose, while Hayashida and Portman used an 0.88 M concentration, thus introducing a considerable change in osmotic value in the mitochondrial surroundings.

In preliminary experiments we could not detect any differences in swelling between mitochondria from EFA-deficient and normal rats when using Johnson's conditions. However, the system used by Hayashida and Portman seemed to be more promising.

In this investigation we therefore first examined the effect of sucrose concentration on the spontaneous swelling of normal and EFA-deficient mitochondria. After suitable conditions had been established, the effect of various doses of sunflower seed oil on mitochondrial swelling was ascertained. Finally the EFA-activity of several unnatural fatty acids was compared, using either body weight or mitochondrial swelling as a criterion.

MATERIALS AND METHODS

Liver mitochondria were prepared from adult Wistar albino rats fed either normal diets or diets deficient in EFA. Drinking water was not limited.

Immediately after decapitation, the livers were removed, cooled on ice and weighed. The entire subsequent isolation procedure was carried out at 0 C. A known amount of the livers was minced and homogenized in a 10-fold volume of sucrose solutions of various concentrations, buffered at pH 7.4 with 0.02 M Tris, and containing 0.001 M EDTA, unless otherwise mentioned. The homogenate was centrifuged for 15 min at 900 x g to remove nuclei and cell debris. The mitochondria were sedimented at 10^4 x g, washed with the same sucrose buffer, re-centrifuged, and suspended in a stock solution of the same sucrose and Tris concentration, but without EDTA. The volume of the buffer was chosen such that 0.1 ml of the stock suspension in 3 ml of the test solution (0.154 M KCl,pH 7.4 with 0.02 M Tris) gave an optical density of 0.7 at 524 nm.

The change in optical density, at 524 nm, of the suspension under examination was taken as the criterion for swelling. The temperature was kept at 25 C by means of a double-walled colorimeter adaptor connected to a thermostat.

The effect of sunflower seed oil on mitochondrial swelling was studied at the end of an EFA bioassay according to Thomasson (3). One hundred and sixty weanling rats were fed a fat-free diet during the whole experiment. After two weeks on this diet, drinking water was limited to 14 ml per day. After five weeks the rats were divided into groups of 10 animals. Each group was intubated five times a week with either 0, 5, 10, 20, 30, 40, 50, 80 or 180

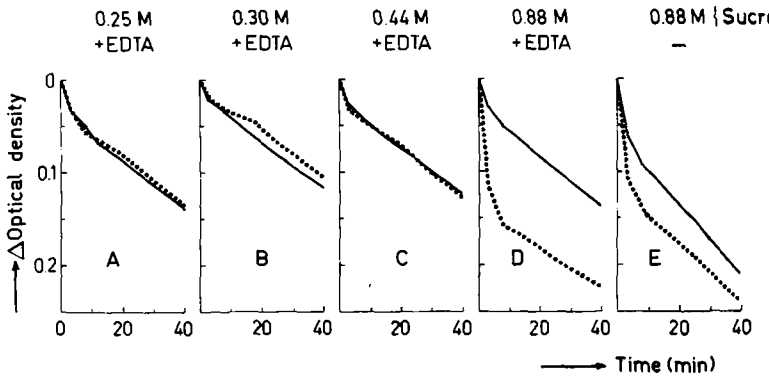

FIG. 1. Effect of sucrose concentration on spontaneous swelling in vitro of liver mitochondria from normal (———) and EFA-deficient (. . . .) rats.

mg sunflower seed oil (linoleic acid content 63%), made up to 0.2 ml with hardened coconut oil.

In the same bioassay the effect of the following synthetic fatty acid methyl esters on the growth rate and mitochondrial swelling was investigated [The unnatural fatty acids were prepared in the Department of Organic Chemistry of our laboratory (4)]: 18:3 ω4 (36 mg/day), 19:3 ω5 (22 mg/day), 20:4 ω6 (10 mg/day, ethylester), 21:4 ω7 (16 mg/day), 22:3 ω8 (26 mg/day). The change in body weight was recorded five times a week, while during the fourth and fifth week of the experiment the mitochondrial swelling was established as described above. By comparing the effects of the fatty acids with those of the various doses of sunflower seed oil, the EFA activity of the former could be calculated for both body weight and mitochondrial swelling.

RESULTS AND DISCUSSION

Effect of Sucrose Concentration on Swelling of Normal and EFA-Deficient Mitochondria

The swelling of normal mitochondria is not affected by the sucrose concentrations used, provided that EDTA is present in the medium (Fig. 1). Omission of this compound (Fig. 1E) increases the swelling because some Ca^{++} ions are probably present, which are known to be very potent swelling agents. At low sucrose concentrations, the swelling behavior of EFA-deficient mitochondria does not differ appreciably from that of normal mitochondria. In the first instance this seems to be contradictory to the results of Johnson, who did find differences in swelling behavior between normal and EFA-deficient mitochondria when prepared in 0.30 M sucrose. However, he also reported that EDTA, present in the medium throughout the process of isolation of the mitochondria, prevented the swelling of both deficient and normal mitochondria to a large extent. It seems therefore possible that deficient mitochondria are more sensitive to small amounts of Ca, which are present when EDTA is omitted (Fig. 1E), than are normal ones.

At 0.88 M sucrose an increase in swelling of the EFA-deficient mitochondria is observed, which is not further stimulated in the absence of EDTA. This is not due to the difference in osmotic value between stock solution (0.88 osmol) and test solution (0.31 osmol), because the results are the same when 0.44 M KCl (0.88 osmol) is used as test solution. Moreover, the permeation of water is so fast as to escape observation in our experimental conditions.

It thus seems that the differences in spontaneous swelling between EFA-deficient and normal mitochondria are best studied when the isolation is carried out at high sucrose concentrations with EDTA (Fig. 1D). This condition has therefore been fulfilled throughout the following experiments.

Effect of Sunflower Seed Oil on Mitochondrial Swelling

The effect of feeding various doses of sunflower seed oil on mitochondrial swelling is shown in Figure 2. Obviously there is a pronounced correlation between the amount of dietary EFA and the extent of the swelling.

The doses of sunflower seed oil have reached their maximum effect after three weeks of feeding, as there is no significant difference in swelling properties among the mitochondria of the rats within 1 dietary group, whether they were measured on the first day of the fourth or the last day of the fifth experimental week.

Close inspection of the average curves of each group reveals that they can be described

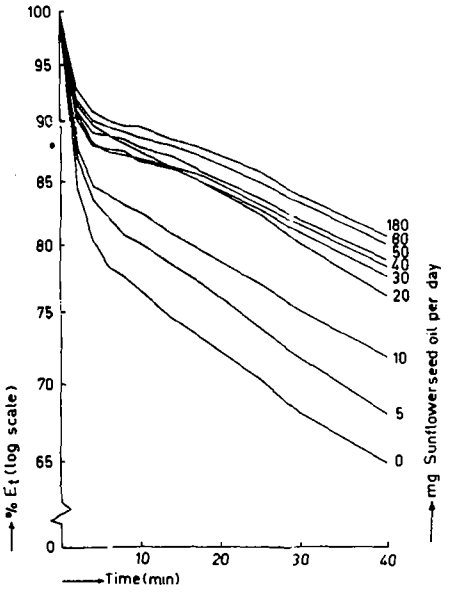

FIG. 2. Recovery of mitochondrial swelling on feeding sunflower seed oil to EFA-deficient rats for 20 days.

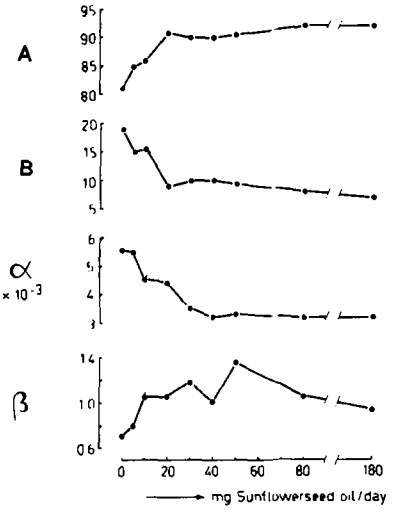

FIG. 3. Relation between parameters A, B, α and β derived from E_t (%) = A . $e^{-\alpha t}$ + B . $e^{-\beta t}$ - and dose of sunflower seed oil.

mathematically by the sum of two exponential functions, the general equation of which is:

$$E_t\ (\%) = A\ .\ e^{-\alpha t} + B\ .\ e^{-\beta t},$$

where E_t (%) is extinction at time t, expressed as a percentage of the initially measured extinction, i.e., the extinction at 15 sec after the addition of the mitochondria to the test solution (The correlation between the observed and calculated curves was invariably better than 99%).

The equation suggests that 2 swelling processes, a fast and a slow one, occur at the same time, each described by 1 term. If the percentage extinction is plotted on a semi-logarithmic scale, as in Figure 2, 2 straight lines are obtained by extrapolation and subtraction, the slopes of which are given by α and β respectively. The parameters A and B are interrelated because of the marginal condition of A + B = 100 at the start of the experiment.

With the aid of an IBM-1800 computer all four parameters were calculated for the individual swelling curves. In Figure 3 these parameters, A and α for the slowly descending part, and B and β for the steep part, are plotted as a function of the dose of sunflower seed oil. They seem to be correlated more or less with the dose applied, although the course of β in this experiment is somewhat erratic. This is probably due to only a few readings being made during the first steep part of the curve. In a subsequent experiment this part of the curve was observed more closely, resulting in values for β that showed much less variation.

Doses of 0.20 mg sunflower seed oil induced changes in the parameters A and B to such an extent that at 20 mg these parameters had reached values which are also observed with normal mitochondria. Parameter α, on the other hand, reacted to a wider range of EFA

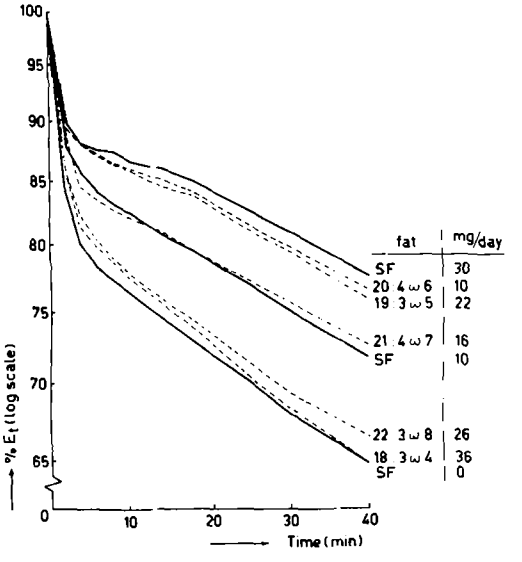

FIG. 4. Recovery of mitochondrial swelling on feeding various synthetic polyunsaturated fatty acids.

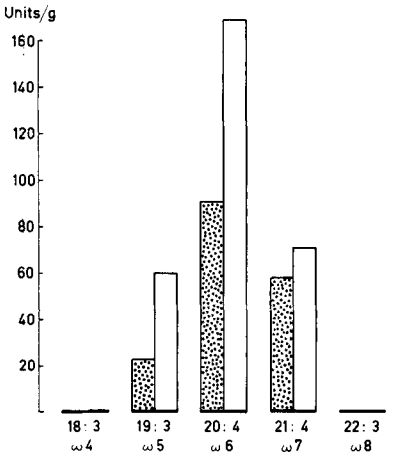

FIG. 5. Comparison of body weight (▨) and mitochondrial swelling (□) as a criterion for EFA activity, relative to linoleic acid (= 100 units/g).

doses (0-40 mg/day, Fig. 3), and was therefore considered the most suitable criterion in a bioassay of essential fatty acids.

Effect of Some Synthetic Fatty Acids on Mitochondrial Swelling

In Figure 4 some synthetic fatty acids are compared with sunflower seed oil for their effect on mitochondrial swelling. It appears that 18:3 ω4 and 22:3 ω8 have no significant EFA activity, whereas the EFA activity of 19:3 ω5, 20:4 ω6 and 21:4 ω7 is considerable. The parameters of the average swelling curves of each of the fatty acids (Fig. 4) are given in Table I.

Using parameter α as a criterion, the EFA activity was calculated, taking into account the amount of fatty acid ingested. In Figure 5 these activities are compared with the results obtained when body weight is used as a criterion. Qualitatively both methods agree very well in that 2 fatty acids have no activity in either criterion and 3 are active in both criteria. However, quantitatively, the mitochondria react more strongly to the 3 active fatty acids than does the body weight.

Similar results were obtained in another series of experiments, in which 2 more fatty acids were assayed, viz., 20:3 ω6-dihomo-γ-linolenic acid, belonging to the linoleic acid family, and 20:3 ω7, another synthetic fatty acid. The second, unnatural one, had no EFA activity with regard to body weight and swelling behavior. Dihomo-γ-linolenic acid, on the other hand, caused an improvement in both criteria, although here again the activity calculated from α was higher than that obtained when the growth rate was used as a criterion.

TABLE I

Parameters of the Swelling Curves of Synthetic Fatty Acids[a]

Fatty acid		Parameter			
Type	Dose (mg/day)	A	$\alpha 10^{-3}$	B	β
18:3ω4	36	83	6.2	18	0.7606
19:3ω5	22	90	4.0	12	1.3077
20:4ω6	10	90	3.8	10	1.2787
21:4ω7	16	86	4.1	15	0.8819
22:3ω8	26	82	5.5	16	0.5692

[a]Calculated from the equation: E_t (%) = A . $e^{-\alpha t}$ + B . $e^{-\beta t}$

The reason for this remains to be investigated.

The results of the experiments indicate that the spontaneous swelling in vitro of rat liver mitochondria when isolated in strongly hypertonic sucrose can be used as a criterion in a bioassay of essential fatty acids, because: there is a correlation between the amount of linoleic acid administered and the degree of recovery of the mitochondrial swelling; fatty acids which have a positive effect on the body weight of EFA-deficient rats also restore normal swelling in the mitochondria; and polyunsaturated fatty acids, as far as investigated, which have no effect on body weight do not affect mitochondrial swelling either.

Advantages of the present method over the bioassay according to Thomasson (3) seem to be that: smaller quantities of fatty acids are needed, because the method seems to be the more sensitive; the bioassay can be performed with rats of any age without the necessity of drinking water restriction; and finally the maximum response is obtained sooner if the mitochondrial swelling is used as a criterion instead of the growth rate. This is sustained by the results of some preliminary experiments with high doses of safflower oil, which indicated that as soon as 1 day after the intubation of 0.5 ml safflower oil to EFA-deficient rats the mitochondrial swelling had been restored to normal.

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[Received January 20, 1969]

Biosynthesis of Fatty Acids in Cell-Free Homogenates of Lactating Gerbil Mammary Gland

JOHN G. CONIGLIO and **RAYMOND B. BRIDGES**, Department of Biochemistry, School of Medicine, Vanderbilt University, Nashville, Tennessee 37203

ABSTRACT

Cell-free homogenates of lactating mammary gland of gerbils maintained on a diet of sunflower seed, guinea pig chow and oats (Diet 1) or a diet of guinea pig chow and oats (Diet 2) and of rats maintained on laboratory chow (Diet 3) were incubated with ^{14}C-labeled acetate, acetyl CoA or malonyl CoA aerobically. A large proportion of the ^{14}C from ^{14}C-acetate and ^{14}C-acetyl CoA incorporated into fatty acids by homogenates from gerbils on Diet 1 was in unsaturated compounds, particularly in 18-carbon and 20-carbon dienoic acids, compared to preparations from animals on Diets 2 or 3. The two radioactive dienoic acids were proven to be $\Delta^{9,12}$ 18:2 and $\Delta^{11,14}$ 20:2, and the latter was shown to be a direct elongation product of $\Delta^{9,12}$ 18:2 by the substrate ^{14}C-acetyl CoA. In all experiments ^{14}C from ^{14}C-malonyl CoA was incorporated predominantly into 14:0 and 16:0, and very little incorporation occurred into unsaturated fatty acids in homogenates made either from gerbil or rat. Total fatty acids isolated from homogenates and from milk fat (fat floating on the centrifuged homogenates) of gerbils on Diet 1 had a higher proportion of 18:2 than animals on the other two diets, a reflection of the large dietary intake of linoleic acid by gerbils on Diet 1. Under these conditions the amount of 18:2 in the mammary gland had a significant effect on the products of the incubation.

INTRODUCTION

Several studies on lipid metabolism in the Mongolian gerbil have been reported (1-6), and it is apparent that this mammal has become useful as an experimental animal in this area. Because of continuing interest in this laboratory in problems of lipogenesis and fatty acid interconversions in various species, a series of investigations in lipid metabolism in the gerbil has been initiated. In this paper we report experiments on incorporation of ^{14}C-substrates into fatty acids by cell-free homogenates of lactating mammary gland of gerbil maintained on two natural diets and a preliminary comparison with similar experiments with rats.

MATERIALS AND METHODS

Animal Studies

Young adult Mongolian gerbils bred and raised in our laboratory were maintained on a diet composed of equal parts of Purina guinea pig chow, oats and sunflower seed. Drinking water was not provided but fresh lettuce was furnished daily. On the day of birth of the pups some of the mother gerbils were kept on the diet described above (Diet 1) and some were placed on a diet composed only of equal parts of Purina guinea pig chow and oats (Diet 2). Rats used in these studies were young adult Sprague-Dawley animals maintained in our laboratory on Purina rat chow (Diet 3). Gerbils or rats were killed 10 to 18 days after parturition, and cell-free homogenates of mammary gland were prepared as described previously (7). The homogenates, without further separation of subcellular fractions, were incubated with the amounts of cofactors used previously with mammary gland preparations made from rats (7). The amounts of cofactors were kept the same irrespective of ^{14}C-substrate used, and the final volume incubated was 1.5 ml. Each incubation flask contained 30-35 mg protein and one of the following substrates: 1-^{14}C-acetate, 5 μc (acetate concentration, 2.5 μ moles), 1-^{14}C-acetyl CoA, 0.5 μc (20 mμ moles), or 1,3-^{14}C-malonyl CoA, 0.25 μc (30 mμ moles). (^{14}C-labeled compounds from New England Nuclear Corporation.) Acetate concentration was kept at 10 mM regardless of ^{14}C-substrate used. Incubations were done at 37 C in air for a period of 1 hr.

Analyses. At the end of the incubation period the reaction vessels were chilled in ice. 1 ml of 40% potassium hydroxide and 0.2 ml of 0.5% hydroquinone (anti-oxidant) were added, and the lipids were saponified under an atmosphere of nitrogen. After acidification of the chilled hydrolysate, fatty acids were extracted with petroleum ether and an aliquot used for determination of ^{14}C-activity by liquid scintillation spectrometry. For determination of ^{14}C-activity in short and medium chain acids, a solution containing 0.2 mg each of C_6, C_8, C_{10} and C_{12} fatty acids was added to an aliquot of the extracted fatty acids, and the mixture injected as free acids onto an 8 ft, 4 mm o.d. column packed with 12% diethylene glycol succinate on 100/120-mesh Anakrom ABS (Analab, Inc., Hamden, Conn.). Temperature of

TABLE I

Distribution of ^{14}C in Total Fatty Acids of Mammary Gland of Gerbils Incubated With Various Substrates

	Diet 1[a]			Diet 2[b]	
Fatty acid	^{14}C-acetate (4)[c]	^{14}C-acetyl CoA (7)	^{14}C-malonyl CoA (4)	^{14}C-acetate (6)	^{14}C-acetyl CoA (6)
6:0	5.3 ± 0.9[d]	5.8 ± 1.0	---	3.8 ± 0.7	4.3 ± 0.9
8:0	6.9 ± 1.5	5.8 ± 0.7	---	6.6 ± 1.1	7.1 ± 1.1
10:0	6.0 ± 1.2	8.3 ± 0.6	0.9 ± 0.4	6.5 ± 1.0	8.4 ± 1.0
12:0	9.7 ± 0.2	9.2 ± 0.4	2.5 ± 1.4	10.1 ± 1.1	11.5 ± 0.9
14:0	16.6 ± 4.5	22.5 ± 3.2	17.1 ± 3.5	22.9 ± 1.0	25.8 ± 2.9
16:0[e]	11.4 ± 1.4	9.7 ± 1.2	63.8 ± 5.2	15.7 ± 2.3	12.1 ± 2.1
18:0	3.0 ± 0.5	2.0 ± 0.5	2.5 ± 0.5	6.3 ± 0.6	3.5 ± 0.5
18:1	4.1 ± 0.1	2.7 ± 0.5	0.9 ± 0.1	9.1 ± 1.4	8.9 ± 1.3
18:2	18.3 ± 5.8	5.9 ± 1.6	1.6 ± 0.2	2.1 ± 0.6	2.0 ± 0.8
20:2	10.8 ± 1.5	15.1 ± 3.5	1.4 ± 0.2	6.9 ± 1.5	4.7 ± 1.2
>20:2	6.2 ± 1.6	7.4. ± 1.1	3.2 ± 0.5	4.0 ± 1.0	3.0 ± 1.0

[a]Guinea pig chow, oats and sunflower seed.

[b]Guinea pig chow and oats.

[c]Number of animals.

[d]Mean ± standard error.

[e]Includes minor amount of 16:1.

the thermal conductivity detector was 240 C, of the injector 270 C and that of the column programmed from 150 to 230 C at 8 °/min. The effluent from the detector passed directly through a heated tube to a proportional counter (Nuclear Chicago Model 4998) kept at 250 C, and the radioactivity monitored simultaneously with the mass signal on a recorder operated in conjunction with a count rate meter. Digital data were also obtained by use of an integrator connected to the count rate meter. ^{14}C-Activity of fatty acids of chain length 6 through 12 carbons was obtained in this fashion. Methyl esters of another aliquot of the extracted acids were prepared by the method of Metcalfe and Schmitz (8) and these were subjected to gas liquid radiochromatography either isothermally or with temperature programming, or in some cases with both techniques, using continuous monitoring as described above. Proper factors were determined by using known standard mixtures of free fatty acids for chain lengths 6 through 12 for free fatty acids, and methyl esters for 12 through 18 carbons (saturated, mono-unsaturated and di-unsaturated), to correct for variations in efficiency of the monitoring system depending on retention time. Lauric acid was used as the fatty acid common to the two procedures, and it was used as a standard both as free acid and as methyl ester. Fatty acids in the diets were extracted by two methods: complete saponification of the fat by refluxing with potassium ɜ, followed by petroleum ether ex- of the acidified solution, and ex- ɔf the lipid with ethyl ether in a Soxhlet extractor, followed by saponification of the fat and petroleum ether extraction of the acidified solution.

Analytical gas liquid chromatographic (GLC) determination of fatty acid methyl esters was done using the same type of column described above with an argon or a flame ionization detector. The response of these detectors was checked with known quantitative standards and corrections were made where appropriate.

Methyl esters of fatty acids to be isolated pure for chemical analysis or degradation were chromatographed on a 0.5 in. o.d., 12 ft column packed with the same material described previously. The esters were collected on siliconized glass wool packed into a long glass tube which was heated to 240 C at the end connecting to the exit port of the thermal conductivity cell. Efficiency of such collection of methyl esters of long chain fatty acids was greater than 85%. Collection of volatile, radioactive oxidative cleavage products separated and identified by gas chromatography was done by using U-shaped collection tubes chilled in a dry ice-acetone bath or in liquid nitrogen.

Separation of methyl esters according to number of double bonds was accomplished by column chromatography using silver nitrate-silicic acid packing with the elution schedule of DeVries (9).

Chemical degradation was done by the method of Dauben et al. (10), oxidative ozonolysis by the method of Stoffel (11), reductive ozonolysis by the method of Stein and Nicolaides (12), and periodate-permanganate oxidation by the method of von Rudloff (13).

TABLE II

Effect of Diet on Fatty Acid Composition of Homogenates and of Fat Floating on the Centrifuged Homogenates

	Per cent of total fatty acid					
	Homogenate			Floating fat		
Fatty acid	Diet 1[a]	Diet 2[b]	P[c]	Diet 1	Diet 2	P
14:0	0.8 ± 0.2[d]	2.3 ± 0.6	0.04	0.5 ± 0.1	2.5 ± 0.2	<0.001
16:0	12:5 ± 1.2	29.7 ± 1.5	<0.001	10.5 ± 2.2	28.8 ± 1.0	<0.001
16:1	0.6 ± 0.2	4.8 ± 0.5	<0.001	0.9 ± 0.3	5.7 ± 0.7	0.002
18:0	4.7 ± 1.1	5.4 ± 0.4	n. s.	3.5 ± 0.6	4.4 ± 0.2	n. s.
18:1	19.6 ± 2.6	30.2 ± 2.0	0.04	23.6 ± 2.4	35.7 ± 4.3	0.05
18:2	61.7 ± 4.5	26.4 ± 1.9	0.002	60.3 ± 7.6	21.6 ± 4.4	0.005

[a]Guinea pig chow, oats and sunflower seed.

[b]Guinea pig chow and oats.

[c]Probability, student t test.

[d]Mean of four animals ± standard error.

Hydrogenation was done by the method of Farquhar (14), and the amount of protein was determined by the biuret reaction (15).

RESULTS

Incorporation of ^{14}C Substrates Into Total Fatty Acids

Whole homogenates of mammary gland of gerbils incorporated into fatty acids all ^{14}C substrates incubated with them under aerobic conditions. Under these conditions less than 1% incorporation was observed with ^{14}C-acetate as precursor, while about 5% of the ^{14}C from added ^{14}C-acetyl CoA and about 40-50% of the added ^{14}C-malonyl CoA were incorporated into fatty acids. Preparations from rat mammary gland incubated with the same medium as the gerbil preparation incorporated up to four times as much ^{14}C from acetyl CoA and about 1.5 times as much ^{14}C from malonyl CoA as did preparations from gerbil gland.

Gas liquid radiochromatography (GLR) of the biosynthesized fatty acids showed a relatively large proportion of the incorporated activity in compounds having retention times equivalent to those of unsaturated fatty acids (Table I) when the ^{14}C-substrate was either acetate or acetyl CoA. Two peaks with retention times corresponding to 18:2 and 20:2 contained the greatest proportion of the ^{14}C-activity of the unsaturated fractions. When ^{14}C-acetate was the substrate, slightly more ^{14}C-activity appeared to be present in 18:2 than in 20:2. However, due to variation in experiments done in vitro, because of difficulties inherent in the technique of GLR and the small number of samples, the difference was not statistically significant at a P value of 0.05. Incubation with ^{14}C-malonyl CoA resulted in major labeling in 14:0 and 16:0 with only small amounts of activity in unsaturated fatty acids and in other saturated acids.

Total fatty acids isolated from homogenates of mammary gland prepared from gerbils on this diet had an extremely large proportion of 18:2 (about 61% of the total fatty acids), presumably linoleic acid. The pattern of fatty acid distribution in these homogenates is shown in Table II. The fatty acids in the top fat obtained with centrifugation (milk fat) are also shown in Table II. The similarity in quantities of each acid in homogenate and in milk fat is obvious and suggests that the homogenate usually contained enough milk fat to dominate the fatty acid pattern. The large amount of 18:2 in these preparations was probably a reflection of the dietary intake of linoleic acid from sunflower seed. The major fatty acids of this diet are summarized in Table III. Although the content of 18:2 was about 53% of total fatty acids, about 70% of sunflower seed fat was 18:2, and the gerbils may have discriminated in favor of this portion of the diet.

Experiments were also done with preparations made from mammary gland of gerbils on a diet of guinea pig chow and oats (Diet 2). The fat of this diet, shown in Table III, had a lower content of 18:2 (about 32%) than did Diet 1. The results of the incubation experiments are shown in Table I. Using ^{14}C-acetate as substrate, smaller amounts of ^{14}C were incorporated into 18:2 (P=0.005) than in the experiments with Diet 1. A smaller amount of ^{14}C was observed also in 20:2, but this was not statistically significant. Corresponding statistically significant increases in ^{14}C-activity with Diet 2 occurred in 18:0 and 18:1 (P=0.001 and 0.02, respectively). With ^{14}C-acetyl CoA the preparations from Diet 2 incorporated less ^{14}C

TABLE III

Total Lipid Content and Fatty Acid Composition of Diets

Diet	Total lipid (g/100 g)	Per cent of total fatty acids							
		<14:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3
Guinea pig chow; oats; sunflower seed	8.5	Trace[a]	0.2	11.0	0.2	2.0	30.1	52.8	3.0
Guinea pig chow; oats	3.2	Trace	1.9	22.9	1.5	6.9	31.6	32.3	3.1
Rat chow	4.6	Trace	3.6	31.8	3.3	9.9	22.7	17.1	---

[a]Trace = <0.1%.

into 18:2 (P=0.05), 20:2 (P=0.01), and compounds of retention times > 20:2 (P=0.02). A statistically significant increase in ^{14}C was observed in 18:1 (P=0.02), but changes in other fatty acids were too small to prove statistically significant. Since no unusual observations had been made with ^{14}C-malonyl CoA as substrate with regard to incorporation into unsaturated fatty acids, only two experiments were done with preparations from animals on Diet 2. Results of the experiments were similar to those using Diet 1.

Identification of 18:2 and 20:2. The relatively large incorporation of ^{14}C-acetate and ^{14}C-acetyl CoA into compounds of retention time equal to 18:2 and 20:2 made it desirable to identify these compounds more rigorously and to try to elucidate the mechanism of their formation. Therefore, material pooled from several incubations was used for the isolation and purification of the compounds by combined techniques of gas liquid and silver nitrate-silicic acid column chromatography. The products were proven to be pure by GLR. The compound with a retention time of 20:2 was hydrogenated and the radioactivity of the hydrogenated compound shown to be associated with a compound of retention time equal to 20:0 by GLR. Confirmation of chain length as 20 carbons and retention time of the non-hydrogenated original compound equivalent to that of 20:2 established the identity of this material as a 20-carbon dienoic acid. The position of the double bonds was established by cleavage of double bonds both by periodate-permanganate oxidation and by reductive ozonolysis. With the latter method essentially all of the ^{14}C originally associated with the 20:2 fraction was now shown by GLR to be in a fragment identified as an 11-carbon ester-aldehyde (carboxyl end of molecule). In the periodate-permanganate oxidation cleavage essentially all of the ^{14}C was shown to be in an 11-carbon dicarboxylic acid (carboxyl end of molecule). This evidence established the first double bond at Δ^{11}. Since double bonds in mammalian fatty acids are methylene-interrupted, the compound must be $\Delta^{11,14}$ 20:2, probably biosynthesized by elongation of 18:2 with a radioactive 2-carbon fragment. This was tested by chemical degradation of the hydrogenated compound to which had been added carrier eicosanoic acid. Ninety-four per cent of the initial radioactivity in the 20:0 was observed to be in the carboxyl carbon which was isolated as benzoic acid. Since the substrate had been carboxyl labeled, the 20:2 apparently was a direct elongation product of $\Delta^{9,12}$ 18:2 and carboxyl labeled acetyl CoA.

The ^{14}C compound with retention time equal to that of 18:2 was also hydrogenated and the ^{14}C-activity was shown to be associated with a compound of 18-carbon chain length. Cleavage of the original compound by periodate-permanganate oxidation was followed by analysis of the radioactive products by GLR. The ^{14}C-activity obtained was low, but it was in a fragment identified as a 9-carbon dicarboxylic acid. This suggested that the original molecule was $\Delta^{9,12}$ 18:2 and, since this was an unexpected result, the analysis was repeated with another batch of pooled biosynthesized 18:2. The degradative procedure used this time, however, was oxidative ozonolysis according to Stoffel (11). Eighty-five per cent of the initial radioactivity was found in the 9-carbon dicarboxylic acid fraction, confirming the original material to be $\Delta^{9,12}$ 18:2. Carbon by carbon degradation was not done.

Comparison With Rats. The incorporation of ^{14}C from acetyl CoA and from malonyl CoA into fatty acids in preparations from gerbils on Diet 1 was compared with similar incorporation in homogenates made from mammary glands of rats maintained on their own laboratory stock diet, i.e., Purina laboratory chow for rats. The fatty acid analysis of this diet is in Table III. Preparations from rats incorporated up to four times as much activity into fatty acids from ^{14}C-acetyl CoA and about 1.5 times as much ^{14}C-activity from malonyl CoA as did preparations from gerbils when the two preparations

TABLE IV

Distribution of ^{14}C in Total Fatty Acids of Mammary Gland of Gerbils and of Rats Incubated With ^{14}C-acetyl CoA or ^{14}C-malonyl CoA

	Per cent of total ^{14}C				
	^{14}C-acetyl CoA			^{14}C-malonyl CoA[b]	
Fatty acid	Gerbil	Rat	P	Gerbil	Rat
8:0	5.0 ± 1.3[a]	4.1 ± 1.6	n. s.	0.9 ± 0.8	1.6 ± 1.0
10:0	6.2 ± 0.7	16.1 ± 4.7	0.05	1.8 ± 1.2	5.9 ± 2.3
12:0	8.9 ± 0.4	34.4 ± 6.2	0.01	4.1 ± 2.1	11.2 ± 2.5
14:0	25.8 ± 4.0	25.7 ± 2.6	n. s.	22.3 ± 5.9	29.3 ± 2.6
16:0, 16:1	10.5 ± 1.0	7.3 ± 2.0	n. s.	52.4 ± 6.9	46.6 ± 9.0
18:0	2.0 ± 0.8	0.7 ± 0.4	n. s.	2.3 ± 1.1	0.9 ± 0.4
18:1	3.3 ± 0.1	0.5 ± 0.2	<0.001	1.4 ± 0.1	0.3 ± 0.2
18:2	3.9 ± 0.8	2.1 ± 1.2	n. s.	3.0 ± 1.0	1.8 ± 1.3
20:2	17.0 ± 6.5	3.6 ± 2.4	0.05	2.4 ± 0.7	Trace
>20:2	8.1 ± 1.4	1.1 ± 0.6	0.01	4.8 ± 1.1	Trace

[a]Mean of four animals ± standard error.

[b]None of these differences is significant.

were used with the same incubation mixture and incubated under the same conditions and at the same time. The distribution of ^{14}C-activity in various fatty acids is shown in Table IV. Relatively more ^{14}C 20:2 was made from ^{14}C-acetyl CoA by the gerbil than by the rat. Moreover, relatively more radioactivity was present in the total unsaturated fraction in the gerbil than in the rat. This was at the expense of the ^{14}C-activity in shorter chain fatty acids. A comparison between gerbils on Diet 2 and the rat revealed significant differences for 10:0, 12:0 and 18:1, as was the case also for gerbils on Diet 1. In addition, the amount incorporated into 18:0 of the gerbil preparation was greater than that for the rat (P=0.05). However, no differences in incorporation into either 18:2 or 20:2 were observed between gerbils on Diet 2 and rats. When ^{14}C-malonyl CoA was used as substrate, little ^{14}C-activity appeared in unsaturated fatty acids in either animal. No statistically significant differences were observed in any of the fatty acids when the amounts could be measured in both groups of animals. Although ^{14}C-activity could not be detected in compounds of retention time greater than 18:2 in rats, it could be detected in the same compounds in the gerbil experiments.

DISCUSSION

The main finding of interest in the studies is the incorporation of ^{14}C from acetate and acetyl CoA into $\Delta^{9,12}$ 18:2 and $\Delta^{11,14}$ 20:2 in the gerbil. This incorporation represented a relatively large part of the total incorporation although the absolute amount may not be considered quantitatively significant. The incorporation of ^{14}C into $\Delta^{9,12}$ 18:2 is interesting in view of the well-known fact that linoleic acid is a dietary essential fatty acid for mammals. Though ^{14}C was incorporated into a fatty acid which was isolated in pure form and the activity shown to be associated with a compound having the structure of $\Delta^{9,12}$ 18:2, no evidence was obtained that this represented net synthesis. It is possible that the ^{14}C was incorporated by an exchange of carbons 1 and 2 of enzyme-bound linoleate with ^{14}C-acetyl CoA, although elongation of some shorter chain precursors by a labeled acetate unit could not be excluded (16). In the laying hen, Reiser et al. (17,18) showed that *cis*-2-octenoic acid-1-^{14}C was converted to labeled linoleic acid appearing in eggs, but Brenner et al. (19) found no evidence for this conversion in fat-deficient rats. That there are certain shorter chain compounds which the rat can convert to linoleic acid was shown by studies of Klenk (20) and of Sprecher (21) in which feeding fat-deficient rats $\Delta^{7,10}$ 16:2 resulted in appreciable conversion to linoleate and long chain metabolites of the linoleic acid family. However, we have no evidence that $\Delta^{7,10}$ 16:2 was present in the diet or in the mammary gland of the gerbil. The presence of ^{14}C-activity in the $\Delta^{9,12}$ isomer of the 18:2 in these studies is in sharp contrast to our findings with rat liver microsomes incubated with ^{14}C-acetyl CoA in which the ^{14}C 18:2 isolated was proven to be the $\Delta^{6,9}$ isomer formed by elongation of a 16-carbon intermediate with 1-^{14}C-acetyl CoA (22). The largest amount of radioactivity accumulated in 18:2 when ^{14}C-acetate was the substrate, although the data did not prove statistically significant (Table I, Diet 1). The reason for this is not known, but it may

be concerned with the slower rate at which acetyl CoA became available when acetate was the labeled substrate. When ^{14}C-acetyl CoA was the labeled substrate furnished it might have been utilized immediately for other reactions (for example, formation of 20:2 from 18:2), leaving relatively smaller quantities for incorporation into 18:2.

The elongation of linoleate to $\Delta^{11,14}$ 20:2 has been reported previously in liver and adrenal tissue (23,24). In our experiment the relative amount of ^{14}C from acetyl CoA incorporated into the $\Delta^{11,14}$ 20:2 was unusually large and apparently was influenced by the large amount of linoleic acid in the tissue. Much less activity was incorporated in the experiments using mammary gland of rats maintained on rat chow and in mammary gland of gerbils maintained on guinea pig chow plus oats (but without linoleic acid). In fact, there were no differences in incorporation into 18:2 and 20:2 between the two latter groups. Apparently, the amount of dietary linoleic acid and hence the amount of this material in the homogenate was the dominating factor in the fate of the ^{14}C-acetate or ^{14}C-acetyl CoA substrate. In homogenates made from rats or from gerbils on Diet 2 the amount of $\Delta^{9,12}$ 18:2 was less than in gerbils on Diet 1, which included sunflower seed. Although the amount of dietary linoleic acid appears to play the dominant role in the incorporation of ^{14}C into 18:2 and 20:2, a difference due to species was not completely ruled out by our experiments. It becomes necessary, therefore, to compare the rat with the gerbil with the two animals on a similar diet, at least with respect to dietary intake of fat. Studies of this type have been hindered by lack of a suitable adequate purified diet for the gerbil which will allow breeding and adequate lactation, but there are now some reports of experiments in which purified diets have been fed to gerbils (6,25).

It has been suggested (26) that in the presence of fatty acids, such as occur in a living system, the pathway of linoleic acid conversion to arachidonic through $\Delta^{6,9,12}$ 18:3 as an intermediate with subsequent elongation to 20 carbons may be preferred over that in which there is first an elongation to $\Delta^{11,14}$ 20:2 with subsequent desaturation. This is based primarily on findings using liver microsomes. In our experiments labeled 18:3 from 18:2 could not have been observed since labeled linoleic acid was not used. However, only traces of 18:3 were observed in the GLC of the homogenate fatty acids from all animals. Furthermore, relatively small amounts of ^{14}C were present in peaks of retention time equivalent to 20:3, the product of elongation of 18:3 by a 2-carbon unit. This would mean that in the experiments with gerbils on Diet 1 either the elongation of 18:2 by acetyl CoA represents a preferred pathway over the desaturation of 18:2 to 18:3 or that it is predominant over the elongation of 18:3 to 20:3.

The use of ^{14}C-malonyl CoA as substrate resulted in most of the label appearing in 14:0 and 16:0 under all conditions used and with both gerbil and rat. The mechanism of the incorporation apparently was different from that of the substrates acetate and acetyl CoA. It is possible that the malonyl CoA was utilized predominantly by the supernatant and the other substrates by the subcellular particles of the homogenate.

ACKNOWLEDGMENTS

Supported by Grants AM-06483 and GM-00300 from the U.S. Public Health Service. R. B. Bridges is a predoctoral trainee under U.S. Public Health Service Grant AM 5441. Technical assistance by Mrs. Shirley Adams.

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[Received February 13, 1969]

Peroxidation of Microsomal Membrane Protein–Lipid Complexes

EDWARD J. VICTORIA[1] and **ALBERT A. BARBER**, Department of Zoology, University of California, Los Angeles, California 90024

ABSTRACT

Nonenzymatic lipid peroxidation was studied using the TBA test on rat liver microsomal fractions, lipid micelles and structural protein-lipid micelle complexes. The kinetics, response to divalent cations, and iron-ascorbate catalysis were alike in the microsomal fraction and in the complex, but different in lipid micelles. The structural protein represented 41% of the total membrane protein, had a $S_{20,obs}$ of 3.5 and was hydrophobic. The binding of lipid micelles by structural protein proceeded in two steps, with an initial fast rate followed by a slower rate. The binding appeared to involve a hyrophobic association between lipid and protein as evidenced by insensitivity to pH, ionic strength and lack of preference for the individual classes of phospholipid micelles. Deoxycholate caused an increase in the initial peroxidation rate in microsomal fractions. Iron and ascorbate catalyzed lipid peroxidation in both the microsomal fraction and in the complex. Iron catalyzed lipid peroxidation but calcium, cobalt and copper inhibited the reaction in the SP-lipid micelle complex. Lipid peroxidation in microsomal suspensions, therefore, appears to be determined, in part, by the hydrophobic nature of the protein-lipid association found in membranes.

INTRODUCTION

The lipid peroxidation reaction in tissue homogenates has been shown to proceed enzymatically (1) or nonenzymatically (2). The reaction in pure lipids differs in several ways from that in homogenates including the kinetics and specificity of metal catalysis (2,3). These differences appear to be due, at least in part, to the association of protein with the lipid in tissue homogenates.

A membrane protein fraction has been isolated from beef heart mitochondria which, because of its noncatalytic nature and relative abundance, has been termed structural protein (SP) (4). Similar proteins have been isolated from several membrane sources (5). These proteins bind to a wide variety of substances, including micellar lipid (6). In this study, the SP-lipid micelle complex was selected as a model system for examining the differences in lipid peroxidation noted between pure lipids and tissue homogenates. Lipids and SP were isolated from the rat liver microsomal fraction. The kinetic behavior, response to divalent cations, and iron-ascorbate catalysis were compared in pure lipid micelles, SP-lipid micelle complexes and microsomal fractions.

MATERIALS AND METHODS

Preparative Procedures

The livers from 6 month old male Sprague-Dawley rats were rapidly removed following stunning, decapitation and exsanguination. They were pooled and homogenized in 0.15 M NaCl, 0.01 M phosphate, pH 7.0 buffer and diluted (1:5 w/v). The homogenate was centrifuged at 1000 x *g* for 10 min. Supernatants were pooled and centrifuged at 10,000 x *g* for 15 min. Supernatants were again pooled and centrifuged at 105,000 x *g* for 90 min. These final pellets were used, without washing, as the microsomal fraction.

Structural protein was prepared by the procedures of Richardson et al. (6) and of Criddle and Fish (7).

Microsomal lipids were prepared by extracting with chloroform-methanol (2:1) by the method of Folch et al. (8). The solvent was removed under nitrogen at 0 C in a modified vacuum microdistillation apparatus. Lipid extracts were stored in the dark at -20 C.

Lipid micelles were prepared by the butanol-cholate method of Fleischer and Klouwen (9). Particulate lipid was removed by centrifugation at 81,000 x *g* for 30 min.

Structural Protein-Lipid Micelle Interaction

The extent of protein-lipid association was determined by measuring the amount of phosphorus bound by SP following incubation with lipid micelles in a Dubnoff metabolic shaker at 37 C. After incubation in 0.001 M EDTA, 0.2 M Tris-acetate, pH 8.0, buffer, the protein-lipid mixture was centrifuged at 1000 x *g* for 10 min. Structural protein and associated lipid sedimented readily while lipid which did not

[1]Present address: Department of Biochemistry, University of Utrecht, Utrecht, the Netherlands.

TABLE I

Amino Acid Composition of Microsomal Structural Protein[a]

Amino acid[b]	μmoles amino acid per 100 mg protein
Lysine	49
Histidine	18
Ammonia (amide NH_2)	74
Arginine	38
Aspartic acid	67
Threonine	40
Serine	45
Glutamic acid	72
Proline	40
Glycine	48
Alanine	51
Half-cystine	8
Valine	50
Methionine	17
Isoleucine	36
Leucine	80
Tyrosine	31
Phenylalanine	42

[a]Results are based on analysis of two separate SP preparations hydrolyzed in vacuum for 22 hr at 110 C in concentrated HCl.

[b]Tryptophan was not determined.

bind to the protein remained in the supernatant. After washing and recentrifuging, the pellets were ashed and their phosphorus content determined (6).

Analytical Methods

Sedimentation coefficients were measured in the Beckman Model E analytical ultracentrifuge as described by Criddle et al. (4). SP was solubilized by a modification of published procedures (4,7) and the solution was concentrated to 1% by dialysis against polyvinylpyrrolidone. Single sector aluminum centerpiece cells or double sector epoxy centerpiece cells were used. The amino acid compositions of two SP preparations were determined in the Beckman Amino Acid Analyzer Model 120 C.

The thin layer chromatographic (TLC) analysis of lipid classes was done as described by De Bohner et al. (10). Ten to 50 μliters of chloroform containing the sample at a concentration of 0.1% to 1.0% were applied to the plate with a microsyringe. The lipid classes were visualized using 0.5% I_2 in chloroform and identified by use of TLC standards as well as by comparison with published results.

DEAE column chromatography of lipids was carried out as described by Rouser et al. (11). The flow rate was 15 ml/min. The polar lipid fractions obtained were evaporated, under vacuum, to 50 ml using a rotary evaporator equipped with a continuous addition adapter and a gas line for the bubbling of nitrogen. The last 50 ml of the sample were evaporated in the modified microdistillation apparatus. Phospholipid classes were checked for purity by TLC and stored at -20 C.

TABLE II

Chemical Analysis of the Microsomal Fraction

Material analyzed	μg/mg protein[a]
Total lipid	389
Total phosphorus	23.3
Lipid phosphorus	15.4
Total phospholipid[b]	370
Total iron	1.25

[a]There were 109 mg protein/g microsomal pellet (wet weight).

[b]Based on the assumption that the average molecular weight was 745 and that the average phosphorus content of the phospholipid was 4.2%.

Gas liquid chromatography (GLC) was used for analysis of fatty acids. Lipid fractions were saponified in methanolic KOH with hydroquinone as antioxidant (12). After extraction, the fatty acids were esterified with BF_3-methanol (13). Samples were dissolved in GLC quality hexane to form a 0.1-2.0% solution and analyzed in a Hewlett-Packard F & M 402 gas chromatograph. Isothermal separation was carried out at 185 C. Fatty acids were identified using NIH-type fatty acid standard kits (14) and by comparison with published results.

TABLE III

Fatty Acid Composition of Microsomal Total Lipid and Phospholipid Fractions[a]

Fatty acid	TL[b]	PC[b]	PE[b]	PI[b]
14:0	0.6	0.6	0.5	1.0
15:0	0.5	0.9	1.0	0.9
16:0	26.8	31.0	25.8	24.2
16:1	2.4	2.9	2.9	3.0
17:0	0.3	0.2	0.5	0.3
18:0	23.8	23.6	30.8	28.4
18:1	10.0	9.5	7.8	9.9
18:2	12.9	12.2	7.8	11.3
20:3	0.3	0.3	0.3	0.4
20:4	17.7	15.1	17.7	16.5
22:5	0.5	---	---	---
22:6	4.3	3.7	4.9	4.0
SA[b]	52.0	56.3	60.6	54.8
UA[b]	48.1	43.7	39.4	45.1
MUFA[b]	12.4	12.4	8.7	12.9
PUFA[b]	35.7	31.3	30.7	32.2

[a]Values are given in mole percentages.

[b]Abbreviations: TL, total lipid fraction; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PI, phosphatidyl inositol; SA, total saturated acids; UA, total unsaturated acids; MUFA, total monounsaturated acids; PUFA, total polyunsaturated acids.

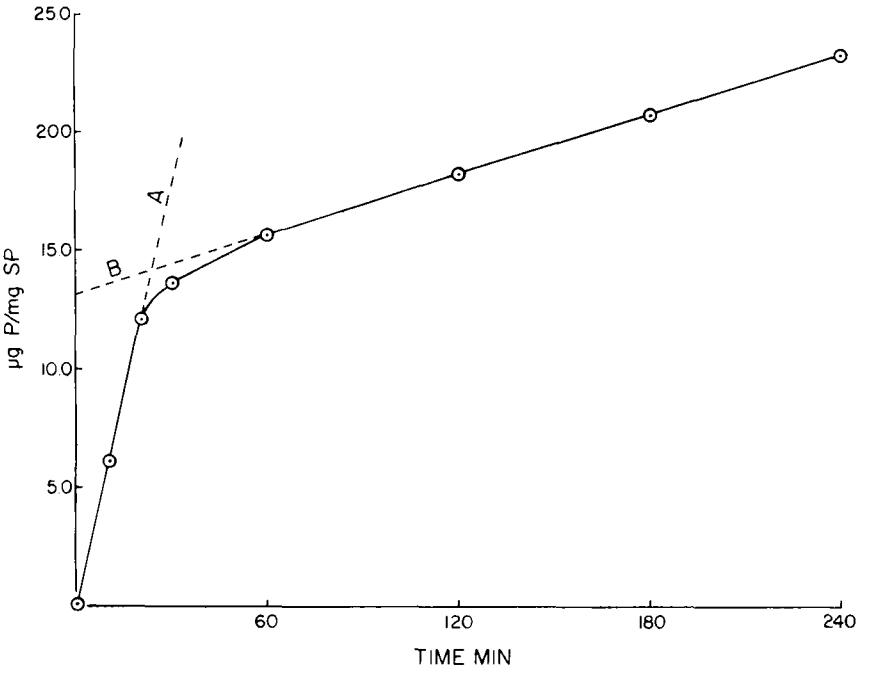

FIG. 1. Binding of microsomal lipid micelles by structural protein. A suspension of 13 ml of SP containing 5 mg protein/ml was incubated at 37 C with 13 ml of lipid micelles containing 100 μg P/ml and 13 ml of 0.005 M dithiothreitol. Reactants were in 0.001 M EDTA, 0.02 M Tris-acetate, pH 8.0 buffer. At the indicated times, 3 ml aliquots were removed and bound lipid determined. A, denotes an initial binding rate of 0.605 μg P/min per mg SP, and B, indicates a subsequent steady binding rate of 0.042 μg P/min per mg SP. Values are the means of triplicate samples.

Area measurements were done by multiplying peak height by width at half height.

Chemical Methods

Protein was determined by the method of Lowry et al. (15) with bovine serum albumin as the standard. Iron was measured using a modification of the *o*-phenanthroline method described by Sandell (16). Phosphorus was measured using a modification of the method of Youngburg and Youngburg (17). Samples were ashed in acid using a sand bath. Color was read at 600 mμ with KH_2PO_4 as the standard. Lipid peroxidation was measured by the thiobarbituric acid (TBA) test as described by Barber (2). TBA values are reported directly as optical density at 530 mμ.

RESULTS

Analysis of Microsomal Structural Protein

The structural protein isolated from the microsomal fraction represented 41% of the total membrane protein. It had an average phosphorus content of 0.5 μg/mg and iron content of 1.0 μg/mg. Sedimentation studies on two SP

TABLE IV

Structural Protein Binding of Micelles From Separate Phospholipid Classes[a]

Phospholipid class	Phospholipid added (μp P/mg SP)	Phospholipid bound (μg P/mg SP)	
		3 min	90 min
Phosphatidyl choline	35	5.6	17.1
Phosphatidyl ethanolamine	35	5.1	15.1
Phosphatidyl inositol	35	6.1	16.4

[a]An SP suspension containing 2 mg protein/ml was incubated at 37 C with equal volumes of lipid micelles at 70 μg P/ml and of 0.005 M DTT. Reactants were in 0.001 M EDTA, 0.02 M Tris-acetate buffer, pH 8.0.

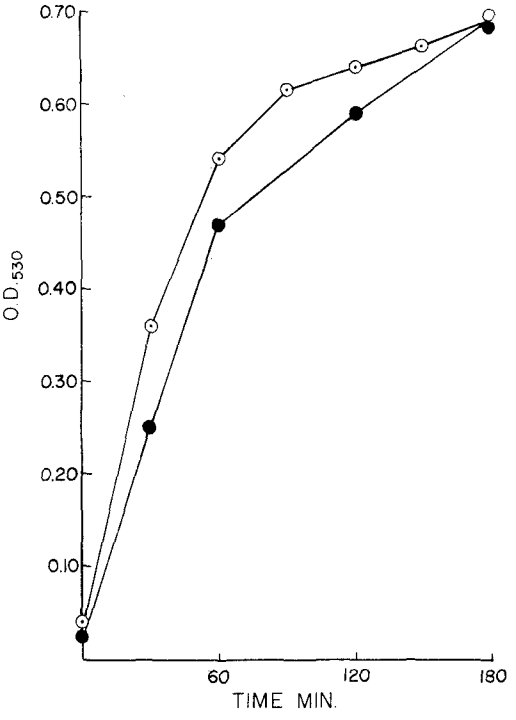

FIG. 2. Lipid peroxidation in microsomal suspensions and SP-lipid micelle complexes. The microsomal suspension, contained 1.5 mg protein, 1.9 μg bound iron, and 23.1 μg lipid phosphorus. The SP-lipid micelle complex contained 1.7 mg SP and 23.3 μg lipid phosphorus; 0.035 mg ascorbic acid was added to each vessel and the final incubation mixture was 4 ml. Reactants were in 0.15 M NaCl, 0.01 M phosphate buffer, pH 7.0. Five micrograms of iron was added to vessels containing the SP-micelle complex. Values are the means of duplicate samples. ●—●, complex; ○—○, microsomal fraction.

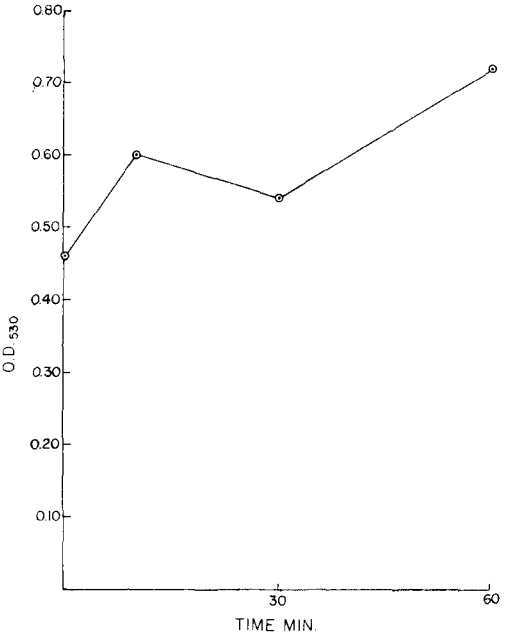

FIG. 3. Peroxidation in lipid micelles. Micelles were dialyzed vs. 0.15 M NaCl, 0.01 M phosphate buffer, pH 7.0. A lipid micelle solution containing 16 μg P was incubated at 37 C with 0.035 mg ascorbic acid and 8 μg iron. Final incubation mixture was 4 ml.

preparations indicated a single schlieren peak with a sedimentation coefficient ($S_{20,obs.}$) of 3.5. Asymmetry of the schlieren pattern indicated heterogeneity. The amino acid composition of the structural protein fraction indicates an average hydrophobicity, as defined by Bigelow (18), of 1153 cal/residue (Table I).

Analysis of the Microsomal Lipid Fraction

The phospholipids comprised 95% of the total microsomal lipids (Table II). The relative distribution of phospholipids, determined as per cent total phosphorus in TLC eluates, was the following: PC, 64%; PE, 24%; PI, 12%. The main microsomal saturated fatty acids were 16:0 and 18:0. Together they comprised about 51% of the fatty acids present (Table III). Arachidonate (20:4) was the most prevalent polyunsaturated fatty acid.

The micellar lipid solutions obtained were faintly opalescent and contained an average of 280 μg P/ml. The phospholipid composition of the micellar solution, as determined by TLC, was identical to that of the microsomal lipid extract used to form the micelles. Thus, no lipid class was preferentially micellized.

Binding of Microsomal Micellar Lipid by Structural Protein

Incubation of microsomal lipid micelles with SP resulted in an initial rapid rate of binding followed by a slower rate which lasted at least 4 hr (Fig. 1). Total amount of lipid bound at 3 and 90 min was similar for the three microsomal phospholipid classes tested (Table IV). Optimal binding occurred in the pH range of 7.0 to 8.0. After the SP-lipid micelle complex was formed, no dissociation of the complex occurred following incubation at pH 5.0 to 9.0 or in 1 M KCl.

Lipid Peroxidation Studies

Time Course of the Reaction. The lipid peroxidation reactions in microsomal fractions, lipid micelles and the SP-lipid micelle complexes were compared. The results for the microsomal fraction and the SP-lipid micelle complex indicate that lipid peroxidation proceeded gradually with an initial rapid reaction

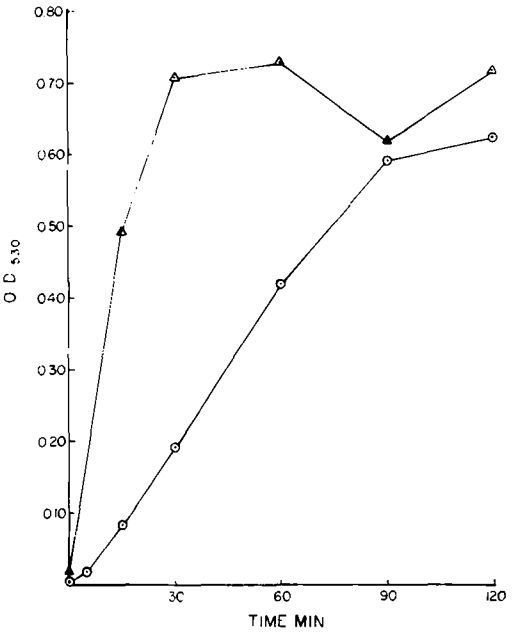

FIG. 4. Effect of deoxycholate on microsomal lipid peroxidation. A microsomal suspension containing 0.40 mg protein was incubated at 37 C with 0.070 mg ascorbic acid and 0.750 mg sodium deoxycholate. Final incubation volume was 4 ml. All reactants were in 0.15 M NaCl, 0.01 M phosphate buffer, pH 7.0. ○------○, control; △------△, DOC.

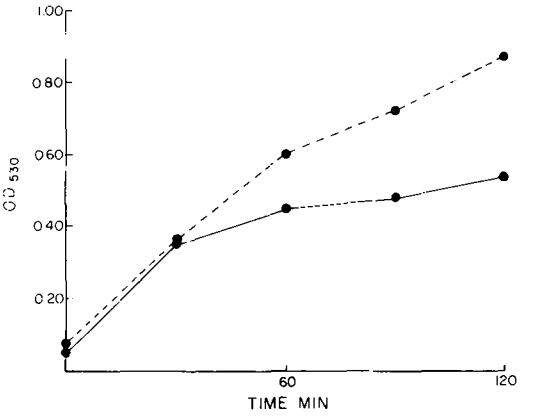

FIG. 5. Effect of ascorbic acid concentration on microsomal lipid peroxidation. A microsomal suspension, containing 1.50 mg protein, was incubated at 37 C in 0.15 M NaCl, 0.01 M phosphate buffer, pH 7.0. Incubation mixture was 4 ml. Ascorbic acid added was 17.5 μg ------, and 280 μg - - - per vessel. Values are the means of duplicate samples.

rate followed by a slower rate (Fig. 2). However, the reaction in lipid micelles was extremely rapid (Fig. 3). The TBA color immediately following the addition of all reactants to micellar preparations was comparable to that achieved only after incubation of the microsomal fraction SP-lipid micelle complex. No TBA color was present prior to the addition of iron. The time course of the lipid peroxidation reaction was altered by the addition of deoxycholate (DOC), a membrane solubilizing agent (Fig. 4). DOC greatly enhanced the initial peroxidation rate. A slight increase in the extent of peroxidation was also noted.

Effect of Divalent Cations. In the SP-lipid micelle complex, iron-ascorbate catalyzed lipid peroxidation was inhibited by the addition of Ca^{++}, Co^{++} and enhanced by the further addition of iron (Table V). The degree of inhibition was greatest with Cu^{++} and least with Ca^{++}.

Effect of Ascorbic Acid. Lipid peroxidation in the microsomal fraction and in the SP-lipid micelle complex was catalyzed by ascorbic acid. Increased amounts of ascorbic acid increased peroxidation in both systems (Fig. 5 and 6). The addition of ascorbic acid to the lipid micelle system had no effect.

DISCUSSION

Analysis of Microsomal Lipids

The results presented confirm that the microsomal fraction is rich in lipid and contains 95% phospholipid and 5% cholesterol. The major phospholipid groups were phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl inositol, as reported by others (19). The microsomal fatty acid composition reported here is also in general agreement with published results (19,20). Of special significance to this investigation was the high content (36%) of polyunsaturated acids found in the microsomal fraction, particularly those with the 1-4, polyene structural feature. These are the fatty acids most susceptible to peroxidation (21).

Chromatographic analysis of lipid micelle components indicated that there was no preferential micellization of any microsomal phospholipid group. The same conclusion has been arrived at on the basis of micelle-DEAE binding studies (22). This result could be expected since the fatty acids of all the phospholipid fractions have a comparable degree of unsaturation.

Analysis of Structural Protein

The protein isolated in the present study fulfilled the criteria commonly used to indicate structural protein. The protein fraction was insoluble over the broad pH range of 2 to 11.

TABLE V

Effect of Divalent Cations on Iron-Ascorbate Catalyzed Lipid Peroxidation in the SP-Lipid Micelle Complex[a]

Cation added	O.D.$_{530}$
None	0.350
1.4 X 10^{-1} μmoles Fe^{++}	0.440
5 X 10^{-3} μmoles Ca^{++}	0.160
5 X 10^{-3} μmoles Co^{++}	0.100
5 X 10^{-3} μmoles Cu^{++}	0.080

[a]A suspension of SP-lipid micelle complex containing 2 mg SP at 21.3 μg P/mg SP was incubated at 37 C in 0.15 M NaCl, 0.01 M phosphate buffer, pH 7.0, with 0.070 mg ascorbic acid, 3.6 X 10^{-2} μmoles iron, and the divalent cations indicated. Incubation volume was 4 ml.

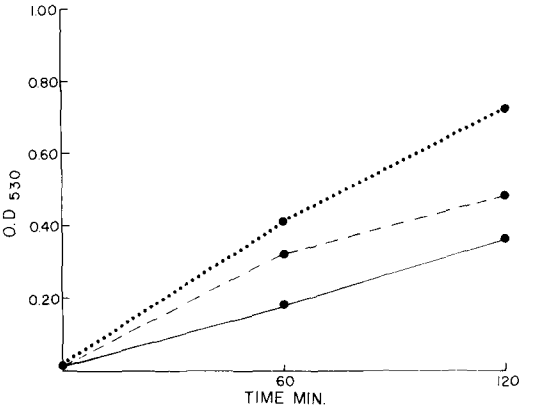

FIG. 6. Effect of ascorbic acid concentration on lipid peroxidation in the SP-lipid micelle complex. A suspension of SP-lipid micelle complex containing 4 mg SP at 6 μg lipid P/mg SP was incubated at 37 C in 0.15 M NaCl, 0.01 M phosphate buffer, pH 7.0. Eight μg of iron was added. Total incubation volume was 4 ml. The ascorbic acid added was 17.5 μg ——, 35 μg - - -, and 70 μg ..... per vessel. Values are the means of duplicate samples.

The amino acid composition indicated an average hydrophobicity of 1153 cal/residue which places it in the top 20% of the over 150 proteins studied (18). Recent evidence suggests that structural protein is not homogeneous but actually represents a group of closely related proteins (19).

Structural proteins bind a variety of biochemical species (5). The binding can be either primarily electrostatic or hydrophobic. It is believed, however, that SP binding to lipid is primarily hydrophobic (24). The liver microsomal SP prepared in this study bound about 45% lipid by weight. This is a higher value than reported for mitochondrial SP (24). The evidence presented is consistent with the idea that the SP-lipid micelle interaction is mainly hydrophobic. This is indicated by insensitivity to pH and ionic strength and by the non-specificity of SP binding to different phospholipid fractions. The microsomal phospholipids isolated can be classified as neutral zwitterionic, mildly acidic and acidic (PC, PE and PI, respectively). The fact that there was no preferential SP binding to any of the three species suggests that the hydrocarbon, rather than the polar moiety of the phospholipid molecule, is involved in the binding. Similar findings have been reported in the literature using other systems (24). The binding reaction proceeded with kinetics similar to that observed in the interaction of mitochondrial SP with lipid micelles (6) and of acetone-extracted mitochondria with lecithin micelles (25).

Lipid Peroxidation Studies

The lipid peroxidation reactions in microsomes and in the SP-lipid micelle complex are similar but different in micelles. A quantitative kinetic analysis of the reactions is not possible now since all three systems contain mixed lipids. Some progress has been made, however, in the kinetic analysis of the autoxidation of single pure fatty acids (26). The similarity between the microsomal and the SP-lipid complex has implications to cytomembrane structure and suggests that, in the membrane, lipid and protein may be associated as in the SP-lipid complex. It is proposed that the nature of lipid peroxidation kinetics in microsomes is a consequence of the mode of lipid protein association. The catalytic action of deoxycholate on microsomal lipid peroxidation suggests that the lipid-protein membrane structure is disrupted in such a way that peroxidation can proceed more rapidly. DOC is known to rupture hydrophobic bonds and thus it is likely that its action results in a rapid unmasking of the peroxidizing sites. A previous study has reported fatty acid binding with several proteins, including serum albumin, gelatin, ovalbumin and diluted horse serum (3). Although binding increased the amount of lipid peroxidation, no effect on the rate of the reaction was noted. The fact that SP does have an effect on lipid peroxidation kinetics that other proteins do not, suggests a very specific role for SP in the structural arrangement of lipid in the membrane.

The effect of divalent cations on lipid peroxidation in pure lipid systems differs from that seen in tissue homogenates (26,27). Whereas cobalt, copper and calcium catalyze peroxidation in pure lipids, they inhibit peroxidation in tissue homogenates and microsomal frac-

tions. The SP-lipid micelle complex shows the same response as tissue homogenates. This evidence also suggests that the SP-lipid complex is similar to the cytomembrane system found in the microsomal fraction.

Ascorbate-iron catalysis has been proposed as the normal nonenzymatic mechanism for lipid peroxidation in tissue homogenates and microsomal fractions (2). Ascorbic acid also stimulates iron-catalyzed lipid peroxidation in the SP-lipid complex system.

It therefore appears, on the basis of the evidence presented, that an in vitro lipid peroxidation system has been developed which closely resembles that of the original intact cytomembrane system. It is likely that many other reactions of cytomembranes can be profitably studied using this same system.

ACKNOWLEDGMENT

Research sponsored by AEC grant AT (11-1), 34 Pro. 49 and by American Cancer Society grant P 422.

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[Received February 10, 1969]

Biosynthesis of Glycolipids: Incorporation of N-Acetyl Galactosamine by a Rat Brain Particulate Preparation[1]

SHIZUO HANDA[2] and **ROBERT M. BURTON**, Department of Pharmacology and Beaumont-May Institute of Neurology, Washington University School of Medicine, St. Louis, Missouri 63110

ABSTRACT

A mixture of UDP-(N-acetyl)glucosamine-1 ^{14}C and UDP-(N-acetyl)galactosamine-1 ^{14}C (7:3) served as a substrate to demonstrate in young rat brain tissue the presence of enzymes which catalyzes the reactions: UDP-glcNAc ⇌ UDP-galNAc (E.C.5.1.3) and UDP-galNAc + gal-glc-cer → galNAc-gal-glc-cer + UDP. The glycolipid acceptor specificity was examined and preliminary kinetic data were obtained for the UDP-(N-acetyl)galactosamine: gal-glc-ceramide N-acetyl galactosamine transferase. The products of the reaction were identified. The relationship of this reaction to ganglioside biosynthesis is discussed.

INTRODUCTION

Gangliosides are a family of sphingosine glycolipids which contain N-acetyl neuraminic acid (1-5). [The abbreviated nomenclature employed for the glycolipids has been described in detail (13)] The major gangliosides possess a common neuraminic acid-free unit consisting of sphingosine, fatty acid (85-95% as stearic acid), glucose, galactose and N-acetyl galactosamine in a molar ratio of 1:1:1:2:1. One or more neuraminic acids are attached to this unit in forming the complete molecule. The structure of a typical dineuraminyl ganglioside is as follows: (N-acetyl)neuraminyl-(2→3)-galactosyl(1→3)-(N-acetyl)galactosaminyl-(1→4)- [(N-acetyl)neuraminyl-(2→3)]-galactosyl-(1→4)-glucosyl ceramide (2-7).

Burton et al. (8) have suggested two possible routes for the biosynthesis of gangliosides. One route is the stepwise addition of monosaccharide units from sugar-nucleotides to ceramide or sphingosine acceptors. The other route is the transfer of an oligosaccharide moiety from a nucleotide to the sphingosine containing acceptor. A combination of both pathways may exist. Evidence in support of the stepwise formation of gangliosides is being accumulated by in vitro experiments in this and other laboratories.

Korey et al (9) and Suzuki (10,11) reported the incorporation of ^{14}C-glucose into gangliosides by a rat brain preparation. Kanfer et al. (12) have shown the incorporation of ^{14}C-N-acetyl neuraminic acid from CMP-neuNAc into gangliosides. Basu et al. (14) reported the biosynthesis of neuNAc-gal-glc-cer by an N-acetyl neuraminyl transferase obtained from the brain of chicken embryo. A similar enzyme which catalizes the transfer of neuNAc from CMP-neuNAc to a tetrahexosyl ceramide to form gal-galNAc-(neuNAc)gal-glc-cer has been described by Arce et al. (15). In addition, Basu et al. (16) and Kaufman et al. (17) have reported the presence of a galactosyl transferase which converts galNAc-(neuNAc)gal-glc-cer to gal-galNAc-(neuNAc)gal-glc-cer and Steigerwald et al. (18) presented evidence for the formation of galNAc-(neuNAc)gal-glc-cer by N-acetyl-galactosamine transferase from the brain of chicken embryo using neuNAc-gal-glc-cer as acceptor.

Hexosamines were used in the present study, because previous in vivo experiments (19) showed that ^{14}C-hexosamine was incorporated almost exclusively into the hexosamine and N-acetyl neuraminic acid moieties of gangliosides, whereas glucose and galactose were incorporated into sphingosine and fatty acids as well as sugar moieties. We wish to report the incorporation of ^{14}C-hexosamine from UDP-(N-acetyl)hexosamine-^{14}C to form galNAc-gal-glc-cer, a possible intermediate in ganglioside biosynthesis, by a cell-free preparation of rat brain. This enzyme preparation contains UDP-(N-acetyl)glucosamine-4'-epimerase (E.C.5.1.3) activity which permits the ready formation of UDP-galNAc.

MATERIALS AND METHODS

UDP-(N-acetyl)hexosamine-^{14}C was prepared in vivo according to Molnar et al. (20) and O'Brien and Neufeld (21). Rat livers (six rats) were removed 15 min after the intraperitoneal injection of 1-^{14}C-glucosamine (New England Nuclear Corp., 8.7 and 9.7 mc/mmole). Nucleotides were extracted from

[1] A preliminary report of this work has been presented orally at the Federation of American Societies for Experimental Biology in 1966 (41).

[2] Fulbright Scholar from Japan. Present address: Department of Biochemistry, Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo, Japan.

TABLE I

Activity of UDP-(N-acetyl)glucosamine-4'-epimerase in Rat Brain Particles[a]

Time min.	N-acetyl glucosamine formed μmoles	Per cent conversion
0	0	0
30	29.0	27.6
60	47.5	45.2

[a]The incubation medium contained a rat brain particle fraction (6 mg protein), 1.05 x 10^{-4} M UDP-galNAc, 3 x 10^{-3} M $MgCl_2$, 2 x 10^{-4} M EDTA, 2 x 10^{-4} M NAD, 5 x 10^{-2} M glycine-NaOH pH 9.0 in a total volume of 1.0 ml. After the incubation at 37 C, aliquots were removed and hydrolyzed with 0.1 N hydrochloric acid at 100 C for 20 min. The aliquots were neutralized and the increase in acetyl-hexosamine color due to the formation of N-acetyl glucosamine, was determined by the method of Reissig et al. (32).

TABLE II

Distribution of UDP-hexNAc:glycolipid N-acetylhexosamine Transferase Activity in Rat Brain Subcellular Particles[a]

Subcellular particulate fraction	^{14}C-N-acetyl hexosamine incorporated, μMKH
H, total homogenate	6.7
R_1, cell debris fraction	6.5
R_2, nuclei fraction	3.3
R_3, nitochondria fraction	7.0
R_4, microsome fraction	7.2
S, supernatant fraction	0

[a]The incubation medium contained 5 x 10^{-2} M potassium phosphate buffer pH 7.6, 5 x 10^{-3} M $MnSO_4$, 500 μg of gal-glc-cer, 5 mg of Triton X-100. UDP-(N-acetyl)hexosamine-^{14}C (1 X 10^5 cpm; 0.2 μmoles) and rat brain particles (6 mg of protein) in a total volume of 1 ml. Subcellular particulate fractions were prepared by the procedure of Brody and Bain (28).

these livers by homogenizing (Waring blendor) in ice-cold 10% trichloroacetic acid. After removal of the precipitate by centrifugation and combining the supernatant fluids from the extract and one wash of the precipitate with cold trichloroacetic acid (550 ml total volume) the trichloroacetic acid was removed from the supernatant by partitioning with diethyl ether (100 ml, five times). The ether in the aqueous supernatant was removed by flushing with nitrogen. The nucleotides were adsorbed on Darco-G-60 (13 g), the Darco collected in a Buchner funnel. The Darco was washed in 0.001 N HCl (100 ml), and eluted with 50% aqueous ethanol containing 0.1% of concentrated ammonium hydroxide (500 ml, 200 ml) by the batch-wise procedure. The eluate was at pH 7.2. The nucleotides were precipitated by adding 25% barium acetate (100 ml) and 1200 ml of cold absolute ethanol. After standing for 16 hr at -20 C, the cotton-like precipitate was collected by centrifugation in a refrigerated Lourdes centrifuge. The supernatant was discarded and the precipitate dissolved in water (30 ml). The barium was removed with sodium sulfate (5 ml of 0.1 M), the precipitate washed with water (10 ml) and the supernatant lyophilized. The lyophilized nucleotides were dissolved in 2 ml of water and streaked on a full sheet of 3 MM-Whatman filter paper. Appropriate standards were placed on each end and in the middle of the paper, to identify the nucleotide bands after ascending chromatographing for 21 hr in a 1:5 mixture of 1 M ammonium acetate and absolute ethanol. The nucleotide areas were located by visualization under ultraviolet light (2600 A). One narrow strip of the chromatogram was scanned for radioactivity by the Vanguard strip counter. The UDP-hexosamine band was cut out of the chromatogram and eluted with water by the Markham technique. The eluate was lyophilized to reduce the volume, dissolved in 2 ml of water and Darco-G-60 (200 mg) was added, collected by centrifugation and washed with 0.001 N HCl (5 ml), and eluted with 50% aqueous ethanol containing 0.3% ammonium hydroxide (2 ml, twice). The eluates were combined, lyophilized, and stored in sealed vials at -20 C until used.

A mixture of UDP-(N-acetyl)glucosamine and UDP-(N-acetyl)galactosamine was obtained in a ratio of glcNAc to galNAc of approximately 7 to 3. UDP-(N-acetyl)galactosamine, prepared according to Glaser (22) was a gift from L. Glaser, Washington University Medical School, St. Louis. Specific activity of ^{14}C-UDP-hexNAc was approximately 0.3 μc/μmole. Glycolipids: cerebroside (gal-cer) from beef brain, gal-glc-cer (23) and galNAc-gal-gal-glc-cer (24) from human red blood cells, neuNGly-gal-glc-cer from horse red blood cells (25) neuNGly-neuNGly-gal-glc-cer from cat red blood cells (26) and ceramide-oligosaccharides obtained by the partial hydrolysis of galNAc-gal-gal-glc-cer (27) were the gift of T. Yamakawa, University of Tokyo. Ceramide oligosaccharides also were prepared from beef brain gangliosides by mild acid hydrolysis, i.e., 0.1 N sulfuric acid at 80 C for 1 hr, followed by 0.1 N hydrochloric acid at 100 C for 1 hr, and then purified by silicic acid column chromatography. The rats (Sprague-Dawley strain from Holtzman Co.) were 12 to 15 day old males, since the biosynthesis of gangliosides in vivo is most active in young rats (19).

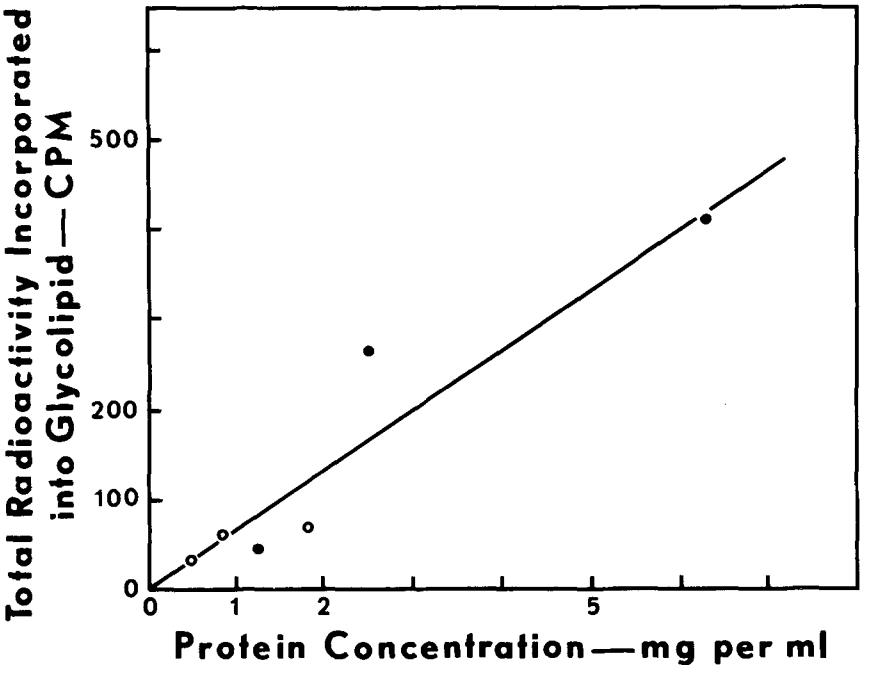

FIG. 1. Effect of enzyme protein concentration on the incorporation of UDP-(N-acetyl)-hexosamine into the glycolipid product. The experimental details are described in Table II, except that the protein concentration was altered as noted. Open and closed circles represent two different experiments.

EXPERIMENTAL PROCEDURES

Rat brains were homogenized with 4 vol of 0.25 M sucrose and separated into subcellular fractions by differential centrifugation according to Brody and Bain (28). As shown in the Results section and Table II, the mitochondrial (R_3) and microsomal (R_4) fractions were the most active and therefore were used in most experiments.

The composition of the basal incubation medium is given in the legend of Table II. The glycolipids in chloroform-methanol solution were added to empty test tubes as measured volumes. The organic solvent was removed by nitrogen and the detergent in aqueous solution was added. Vigorous vibration with the Vortex mixer and gentle warming when needed resulted in a smooth colloid which could be dispersed in the final aqueous solution. The enzyme preparation was always added last to start the reaction. After the incubation period at 37 C (3 hr), the reaction was stopped by the addition of 20 vol of chloroform-methanol (2:1 v/v). The lipids were partitioned by Folch's method (29). Radioactivity in the organic phase (lower) was determined in a Packard Tri-Carb liquid scintillation spectrometer. In general, the aqueous phase (upper) had only negligible radioactivity after dialysis. In this procedure, the gangliosides are largely contained in the aqueous phases, while the organic phase contained the non-acidic glycolipids, including the product, galNAc-gal-glc-cer.

In some experiments (Fig. 4) the radioactivity of the glycolipid product was determined by the procedure of Goldfine (30). The reaction mixture was placed on filter paper, dried and washed with ice-cold 10% trichloroacetic acid for 30 min. A 10 min rinse of the filter paper in ice-cold trichloroacetic and two washes in ice water were used. After drying, the radioactivity was first measured in a Beckman LS-133 liquid scintillation spectrometer. After the filter paper was removed from the counting vial, it was extracted with chloroform-methanol (2:1) for 2 hr in a Soxhlet apparatus. The radioactivity was determined for a second time. The difference between the first and second radioactivity measurements indicate radioactivity of the glycolipid product.

Enzyme activities are reported as moles of substrate reacted or product formed per kilogram protein per hour (MKH) under defined conditions. Protein was estimated by the procedure of Lowry et al. (31). N-acetyl glucosamine was measured by the procedure of Reissig et al. (32).

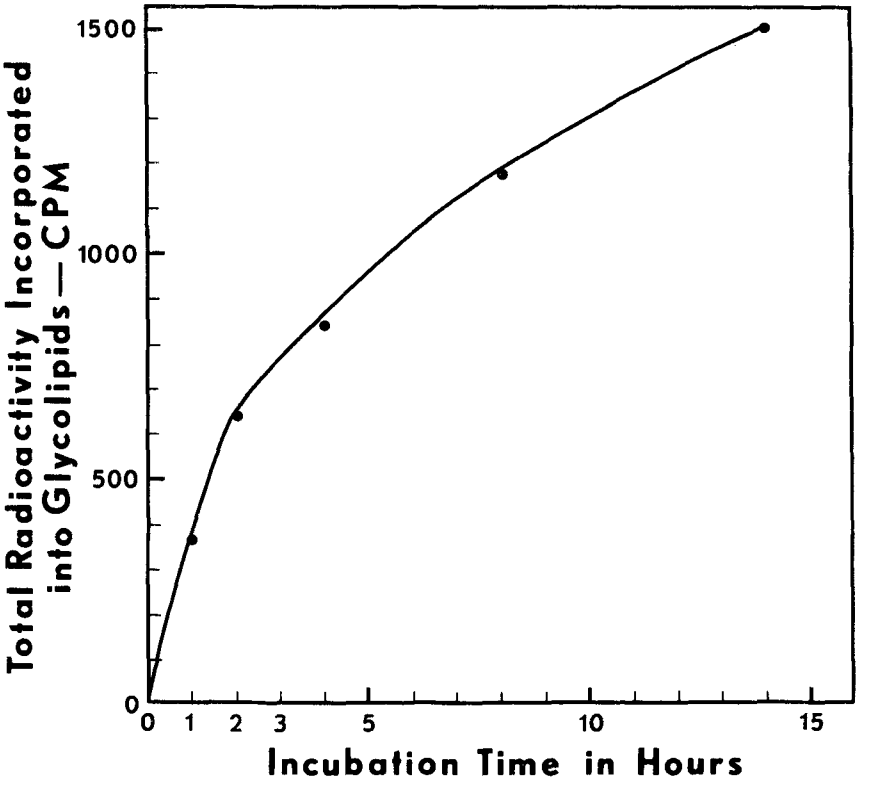

FIG 2. Effect of time on the incorporation of UDP-(N-acetyl) hexosamine into the glycolipid product. The experimental details are described in Table II, except that the time interval of incubation was altered as indicated.

RESULTS

UDP-(N-Acetyl) Glucosamine-4'-Epimerase (E.C.5.1.3.) Activity

Because the substrate used was a mixture of UDP-(N-acetyl)glucosamine-^{14}C and UDP-(N-acetyl)galactosamine-^{14}C, it was necessary to be sure that the rat brain particles contained UDP-glcNAc-4'-epimerase activity. The epimerase activity was assayed according to the procedure of Glaser (22) and, as shown in Table I, rat brain particles do contain UDP-(N-acetyl)glucosamine-4'-epimerase. The epimerase activity of the young rat brain particles was estimated to be 50 mMKH.

Distribution of UDP-hexNAc: Glycolipid N-Acetylhexosamine Transferase Activity in Subcellular Particles

Subcellular particles prepared by the procedure of Brody and Bain (28) were examined for UDP-hexNAc:glycolipid (N-acetyl) hexosamine transferase activity. The data reported in Table II show that all of the particles contain enzyme activity. The mitochondrial (R_3) and microsomal (R_4) fractions contained most of the enzyme activity. However, their specific enzyme activity was not much greater than that of the initial homogenate, suggesting that either the enzyme is labile and partially inactivated during the isolating of the particles or that an essential component of the enzyme is lost during the subcellular fractionations. Experiments proved that the latter possibility is not true because recombining the subcellular fractions resulted only in an additive increase in transferase activity. The particulate nature of the enzyme was demonstrated by the total lack of activity in the supernatant fraction.

Using the basal incubation system with different amounts of enzyme protein, the incorporation of ^{14}C-N-acetyl hexosamine into the glycolipid was proportional to the protein concentration (Fig. 1). Figure 2 indicates that the incorporation of the radioactivity increases with time up to 14 hr, the longest time interval examined.

The Effect of Various Incubation Conditions on the Enzyme Activity

The incorporation of N-acetyl hexosamine into the glycolipid was increased by the addition of detergents. Triton X-100 was the most effective among the detergents tested and it increased the incorporation about 1.8-fold. The detergents examined showed the following order of decreasing activity: Triton X-100, Tween-40, Cutscum, BRIJ-96, Tween-20 and Tween 80.

Divalent cations increased the activity of the UDP-hexNAc:glycolipid transferase system. Manganese ions were most effective with magnesium ions being only 80% effective.

Effect of Substrate Concentrations on the UDP-HexNAc: Glycolipid N-Acetyl Hexosamine Transferase Activity

The data presented in Figure 3 show an increase in transferase activity with increasing UDP-hexNAc concentration. Linearity of the Lineweaver-Burk plot is shown in the insert of Figure 3. Since the UDP-galNAc-4'-epimerase is present in excess, the UDP-galNAc to UDP-glcNAc ratio must be maintained at equilibrium. Thus the concentrations of UDP-galNAc in the reactions mixture can be estimated and a first approximation of the K_m for UDP-galNAc can be estimated. This estimated K_m is 1.2 x 10^{-4} M. The nucleotide product of the transferase reaction was shown to be UDP by measurements with pyruvic acid kinase (E.C.2.7.1.40) and phosphoenol pyruvate.

The lipid acceptor, gal-glc-cer, was employed in the study presented in Figure 4A. It may be seen that greater enzyme activity was obtained with gal-glc-ceramide than with neuNgly-gal-glc-ceramide. The Lineweaver-Burk plot is shown in the insert to Figure 4A. Figure 4B is a photograph of a thin layer chromatogram which shows that gal-glc-cer did not contain neuNgly-gal-glc-cer and that the neuNgly-gal-glc-cer was free of gal-glc-cer.

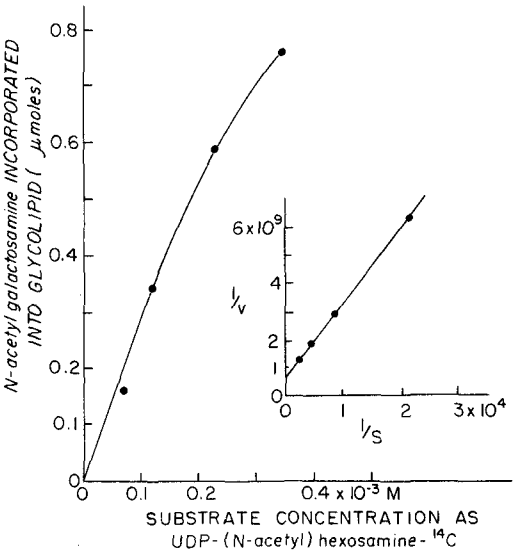

FIG. 3. Effect of UDP-(N-acetyl)hexosamine concentration on incorporation into the glycolipid product. The experimental details are described in Table II and the text, except that the UDP-hexNAc-^{14}C concentration was altered as noted. Results are reported as total μmoles incorporated hexNAc. The insert is a plot of the data according to Lineweaver-Burk (42).

TABLE III

Lipid Acceptor Specificity of the Rat Brain UDP-hexNAc:glycolipid Transferase[a]

Source of glycolipid	Glycolipid acceptor used	^{14}C-N-acetyl hexosamine incorporated, μMKH
Bovine brain	gal-cer[b]	11.1
Gangliosides	glc-cer[c]	10.5
Globoside	glc-cer[b]	8.8
Ganglioside	gal-glc-cer[c]	11.3
Globoside	gal-glc-cer[b]	12.8
Globoside	gal-gal-glc-cer[b]	2.8
Gangliosides	galNAc-gal-glc-cer[c]	3.5
Gangliosides	gal-galNAc-gal-glc-cer[c]	3.5
Globoside	galNAc-gal-gal-glc-cer[b]	2.4
Horse red blood cells	neuNGly-gal-glc-cer[b]	1.6
Cat red blood cells	neuNGly-neuNGly-gal-glc-cer[b]	2.3

[a]Incubation conditions are described in the Experimental section and legend to Table II. All glycolipid acceptors were at concentrations of 500 μg/ml. After incubation, glycolipids were extracted with chloroform-methanol (2:1 v/v) and chromatographed on paper with 0.1% sodium tetraborate. The spot at the origin was cut out and its radioactivity determined.

[b]The fatty acid is primarily 24:0.

[c]The fatty acid is primarily 18:0.

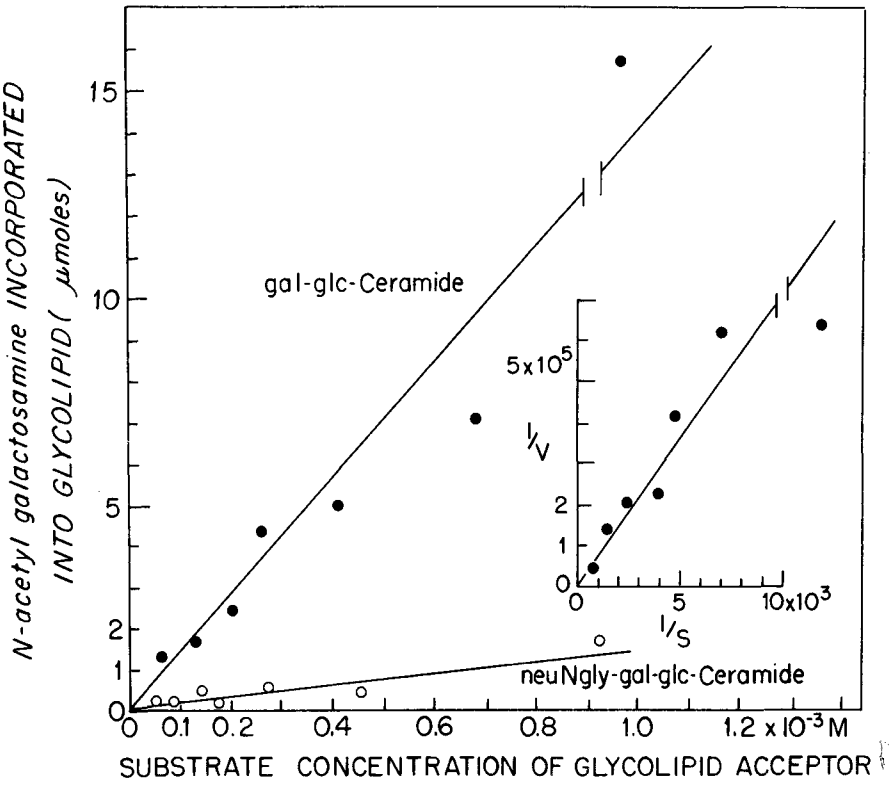

FIG. 4A. Effect of gal-glc-ceramide concentration on N-acetyl hexosamine incorporation into the glycolipid product. The experimental details are described in Table II and the text, except that the glycolipid acceptor concentration was altered as noted, 6.5 x 10^{-3} M UDP-hexNAc was used, and 1 x 10^{-4} M $MgCl_2$ replaced the $MnCl_2$. Results are reported as total μmoles hexNAc incorporated. The insert is a plot of the data according to Lineweaver-Burk (42).

Glycolipid Acceptor Specificity

As shown in Table III, the glycolipid acceptors which contain one or two hexose moieties are better acceptors than the glycolipids which have three or more sugar residues. In addition, both of the neuraminic acid containing glycolipids studied had low activity at the concentration employed. Thus gal-glc-cer is the best acceptor studied being over sixfold more active than neuNGly-gal-glc-cer.

Since brain gangliosides mainly contain 18:0 fatty acids, it was of considerable interest to know whether the chain length of the fatty acid residues affected the ability of the glycolipids to serve as N-acetyl hexosamine acceptors. The glycolipids prepared from animal red blood cells and the cerebrosides from brain have longer fatty acids, centered around 24:0, than those prepared from brain gangliosides. From the data presented in Table III, it may be seen that the size of the fatty acid residue was without significant effect on the incorporation of radioactive N-acetyl hexosamine using either glc-gal-cer or glc-cer as acceptors.

The incorporation of N-acetyl hexosamine into the glycolipid was markedly reduced when high concentrations, i.e., 0.1-0.3 M, of sucrose, lactose, maltose, glucose, galactose, or galactosamine were added to the complete reaction mixture. However, at substrate concentrations (3 x 10^{-4} M), the reaction was not observed to be inhibited by these sugars. Since high concentrations of sucrose are used frequently in the preparation of subcellular particles, the sucrose inhibition can become a technical problem. Because of this, some particle preparations were washed in potassium phosphate buffer to reduce their sucrose content.

Identification of the Reaction Product

The glycolipid formed during the incubation of reaction mixtures (essentially as described in Table II) was dissolved in chloroform-methanol

(2:1) and washed with water. The washed lipids were chromatographed on a silicic acid column, being eluted with mixtures of chloroform-methanol as shown in Figure 5. Most of the radioactivity was eluted in Fractions 7 and 8, the fractions which have been shown to contain galNAc-gal-glc-cer.

Fractions 7 and 8 were combined and examined by thin layer chromatography (TLC) on silica gel plates using a solvent system of chloroform-methanol-water (65:25:4 v/v) as described by Wagner et al (33). The TLC plate was divided into eight areas and the radioactivity of each area determined by placing the silica gel directly in the scintillation fluid. As illustrated in Figure 6, the major radioactive product chromatographed with the same mobility as the reference galNAc-gal-glc-cer. This radioactive glycolipid separated readily from related glycolipids such as gal-glc-cer and glc-cer (Fig. 6) and from the various gangliosides.

The radioactive glycolipid product was dissolved in methanol containing 4% anhydrous hydrogen chloride and heated at 85 C for 17 hr. The methyl esters were extracted with petroleum ether and shown not to be radioactive. After removal of the methyl esters, the methanol phase was separated into the sphingosine and methyl glycoside fractions by TLC (Silica gel, chloroform-methanol-2 N ammonium hydroxide, 40:10:1 v/v/v). The sphingosine so isolated was not radioactive. All of the radioactivity of the glycolipid was recovered in the area near the origin containing methyl glycosides. This radioactive glycoside reacted with ninhydrin indicating it to be a primary amine.

In another experiment, the glycolipid was hydrolyzed with 2 N hydrochloric acid at 100 C for 3 hr. After hydrolysis, the carbohydrates were separated from the lipid moieties by partitioning between chloroform-methanol and water (29). The aqueous phase was concentrated and chromatographed on Whatman No. 1 filter paper (*n*-butanol-pyridine-water, 6:4:3 v/v/v). The radioactivity was found to occur only in the galactosamine spot, which separated well from the hexoses. In addition, glucosamine was not present nor was any radioactivity found in the area where glucosamine would migrate.

The results of these experiments are consistent with the interpretation that radioactive glycolipid product is galNAc-gal-glc-cer, in which the radioactivity resides solely in the galNAc moiety.

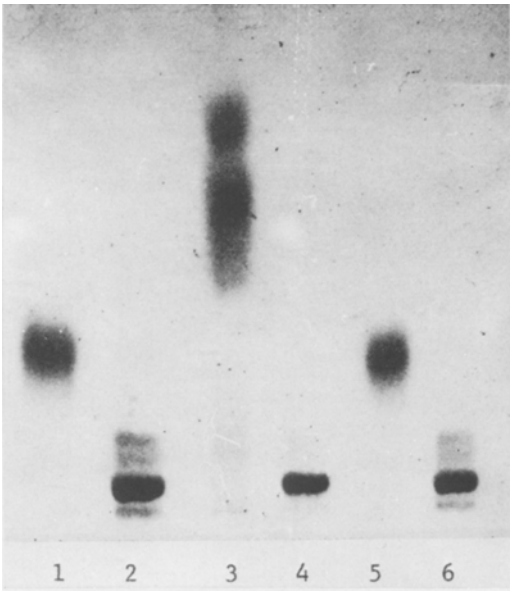

FIG. 4B. Thin layer chromatogram of gal-glc-cer and neuNGly-gal-glc-cer. The glycolipids were obtained as described in Methods. Silica gel G plates (0.25 mm) were developed in chloroform-methanol-water, 65:25:4 v/v/v (33) and the glycolipids located with the anthrone-sulfuric acid spray. Lanes 1,5, gal-glc-cer; lanes 2,6, neuNGly-gal-glc-cer; Lane 3, brain cerebroside mixture; and Lane 4, galNAc-gal-gal-glc-cer. The light bands in Lanes 2 and 6 react with Bial s reagent.

DISCUSSION

The data presented indicate the incorporation of hexosamine from UDP-(N-acetyl)-hexosamine into a glycolipid by rat brain particles. This glycolipid has been identified as galNAc-gal-glc-cer by its mobilities on silicia acid columns and on thin layer chromatograms. Radioactivity was found exclusively in the galactosamine moiety of the glycolipid. Although a mixture of UDP-(N-acetyl)galactosamine and UDP-(N-acetyl)glucosamine was used as the substrate, rat brain particulate fractions contain UDP-(N-acetyl)glucosamine-4'-epimerase activity in sufficient excess to maintain the equilibrium mixture of the nucleotides as the UDP-galNAc is used in glycolipid synthesis.

The data presented in this paper, in addition to other experiments, are consistent with the following reaction sequence for ganglioside biosynthesis in rat brain:

(a) cer + UDP-glc → glc-cer + UDP

(b) UDP-glc ⇌ UDPgal

(c) glc-cer + UDP-gal →
gal-glc-cer + UDP

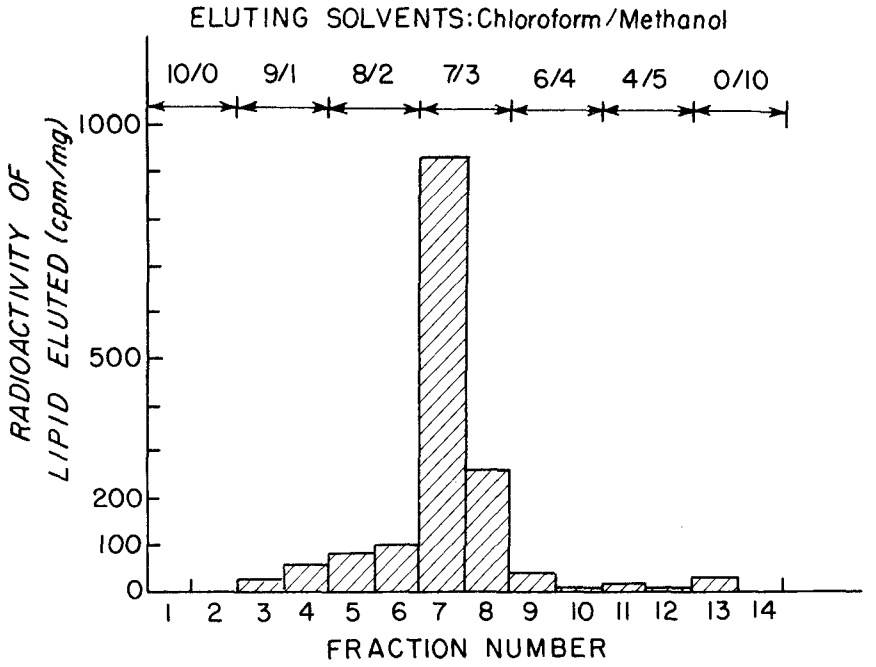

FIG. 5. Silicic acid column chromatography of the radioactive glycolipid product. The radioactive glycolipid product formed and isolated as described in Table II and the text was suspended in chloroform and chromatographed on 4 g of silicic acid by stepwise elution with 40 ml portions of chloroform-methanol mixtures. The solvent mixtures employed are shown in the figure. Reference glycolipids were chromatographed under similar conditions to relate specific glycolipids and their elution patterns.

(d) UDP-glcNAc ⇌ UDP-galNAc

(e) gal-glc-cer + UDP-galNAc →
galNAc-gal-glc-cer + UDP

(f) galNAc-gal-glc-
cer + CMP-neuNAc ⇌
galNAc-(neuNAc)gal-glc-cer + CMP

(g) galNAc-(neuNAc)gal-glc-
cer + UDP-gal →
gal-galNAc-(neuNAc)gal-
glc-cer + UDP

Reaction a has been demonstrated in chicken brain particles by Basu (34) and in rat brain particles in experiments in which UDP-glc was a better hexose donor than UDP-gal (Burton, unpublished data). Experiments conducted essentially as described (37) and using rat brain particles heated to 52 C for 2 min to inactivate the UDP-glucose-4'-epimerase showed the formation of glc-cer. The two lipid acceptors studied were ceramide (N-pal-sph) and sphingosine. BRIJ-96 was used to bring the lipid acceptors into the aqueous incubation mixture. UDP-glc donated glucose to ceramide at a rate four times greater than the endogenous rate. No transfer of the glucose to sphingosine occurred. UDP-gal donated galactose to sphingosine at a rate 17 times greater than the endogenous rate. Ceramide was not an acceptor for galactose. The UDP-glc:ceramide glucosyl transferase was only ¼ as active as the UDP-gal:sphingosine galactosyl transferase. Reaction b has been shown by Maxwell et al. (35,36) and Burton et al. (37) to occur in rat brain and to be heat sensitive, a property which allowed the dissociation of reaction a and b. Reaction c has been demonstrated by Hauser (38) to occur in rat spleen, and Basu (34) has shown this galactosyl transferase to be present in chicken brain particles. This paper presents data which show that reactions d and e can occur. Kanfer et al. (12) have documented reaction f using rat kidney preparations, and their evidence also suggested the occurrence of reaction g. Basu et al. (16). have reported the presence of an enzyme which catalyzes reaction g in chicken embryo brain, and Yiamouyiannis and Dain (39) have found a similar enzyme in frog brain. It is pertinent to note that brain tissue from patients afflicted with infantile amaurotic familial idiocy accumulate galNAc-gal-glc-cer (40) as well as galNAc-(neuNAc)gal-glc-cer

(Tay-Sachs' ganglioside).

The in vitro rate of synthesis of galNAc-gal-glc-cer is about 1.7 μMKH (wet weight tissue) under the conditions described. This rate is sufficient to supply this glycolipid as a possible precursor of gangliosides, which are accumulated in vivo at a rate of 1.5 μMKH (wet weight tissue) in 12- to 14-day-old rats (19). The rate of the UDP-glcNAc-4'-epimerase reaction (50 mMKH) is more than sufficient to provide the UDP-galNAc needed.

There is a difference in the results obtained by Steigerwald et al. (18) for the UDP-galNAc:glycolipid transferase in which the preferred glycolipid substrate was neuNAc-gal-glc-ceramide and the results reported by Handa and Burton (41; this paper) in which the preferred glycolipid substrate appeared to be gal-glc-ceramide. In experiments designed to be identical with those of Steigerwald et al. (18), it was observed that in fact, neuNAc-gal-glc-ceramide accepted approximately 10-fold more N-acetyl galactosamine than gal-glc-ceramide with chick embryo brain particles. Rat brain particles catalyzed a twofold increase in N-acetyl galactosamine incorporation by neuNAc-gal-glc-ceramide over that incorporated by gal-glc-ceramide at concentrations employed by Steigerwald et al. (18). Under the conditions used (i.e., lower glycolipid concentrations) to obtain the data presented in Table III, the gal-glc-ceramide was always the better acceptor. The use of highly purified neuNAc-gal-glc-ceramide as described in Figure 4 resulted in negligible incorporation of N-acetyl galactosamine under conditions in which gal-glc-ceramide was an efficient acceptor. There appeared to be two possible factors involved in explaining the two sets of data, i.e., species difference and the physical properties of the glycolipid acceptors.

The glycolipids used in these experiments are only sparingly soluble in water; therefore, detergents were used to keep the glycolipids in water as colloids. The precise size and shape of the colloidal unit or micelle depends upon the specific glycolipids and the detergent employed. These micelles must have the terminal galactose exposed sufficiently to allow reaction with the nucleotide substrate and the enzyme. The rate and extent of the N-acetyl galactosamine transferase activity was dependent upon the detergent used and Triton X-100 was the most efficient. Some of our experiments suggest that the more polar glycolipid, neuNAc-gal-glc-ceramide, might function in vitro as a detergent to form a micellar solution of gal-glc-ceramide.

Because the substrate for the N-acetyl galactosamine transferase reaction was present as micelles, the kinetics of the incorporation were impossible to interpret being dependent on the factors mentioned above. An estimated K_m for these glycolipids acceptors has little meaning and probably will be different for different detergents. Such experimental problems have been encountered in other studies, for example, the synthesis of rhamnolipids which involved the substrate β-hydroxydecanoyl-β-hydroxydecanoic acid, which probably exists as a micelle since the reaction rate is independent of concentration (43), and studies with palmitoyl-CoA which clearly form micelles (44).

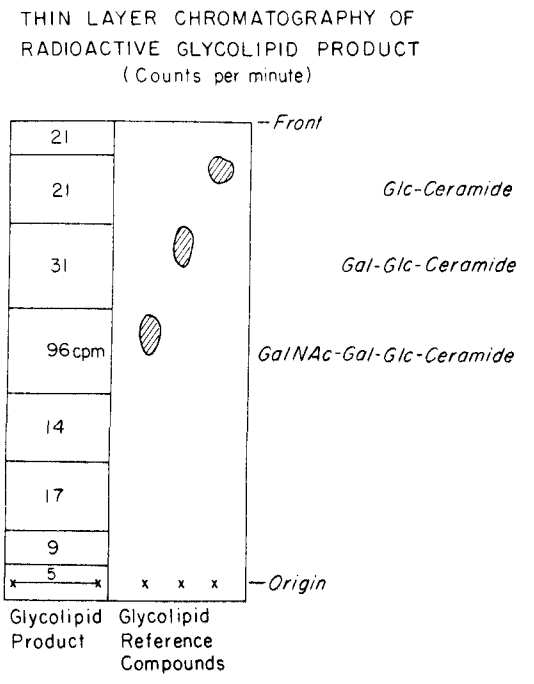

FIG. 6. Thin layer chromatography of the radioactive glycolipid product. The radioactive glycolipid isolated from a silicic acid column, as described in the legend to Figure 5, was chromatographed by TLC using silica gel (Absorbosil-1) and chloroform-methanol-water, 65:25:4 v/v/v (33). After development, the dry plates were exposed to iodine vapor to provide guidelines for sectioning the glycolipid product tract. The silica gel in the eight subdivisions was removed and transferred to vials, 10 ml scintillation fluid (toluene, DPO, POPOP) was added, and the radioactivity measured in a Packard Tri-Carb liquid scintillation spectrometer. The glycolipid reference spots were detected by dichromic-sulfuric acid charring.

ACKNOWLEDGMENT

Supported in part by research grant NB 01575-12 from the National Institute of Neurological Diseases and Stroke, U.S. Public Health Service.

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[Received February 8, 1968]

Species Variations in Phospholipid Class Distribution of Organs: I. Kidney, Liver and Spleen

GEORGE ROUSER, GERALD SIMON[1] and **GENE KRITCHEVSKY**, Section of Lipid Research, Division of Neurosciences, City of Hope Medical Center, Duarte, California 91010

ABSTRACT

Improved procedures for preparation of lipid extracts and determination of phospholipids by phosphorus analysis of spots separated by thin layer chromatography (TLC) were employed to determine the phospholipid class distributions of vertebrate (human, bovine, rat, mouse, frog) kidney, liver and spleen. The absence of significant changes arising from postmortem enzymatic degradation was demonstrated by analysis of lipids of organs after standing for different times postmortem. Intraspecies variability was evaluated by separate analysis of several rat organs. Accuracy of analytical results was insured by demonstrating the absence of spot overlap by two-dimensional TLC and low values for standard deviations. The values for kidney and liver demonstrate little or no species variability, whereas values for spleen indicate two groups which differ in cellular composition. The findings for kidney and liver are in keeping with data obtained from heart, skeletal muscle, lung and highly purified subcellular particulates which indicate that, among vertebrates, there is little or no species variability of phospholipid class distribution of organs and most subcellular particulates.

INTRODUCTION

This report is one of a series in which data are presented for species variations of animal cell membrane phospholipid class distribution as disclosed by analysis of whole organs and highly purified subcellular particulates. Data for kidney, liver and spleen are presented in this report. Data for other organs and subcellular particulates are presented in other reports (1-5).

MATERIALS AND METHODS

Sampling Procedures

Human organs, obtained as soon as possible, were frozen, then maintained at -20 or -70 C until extracted. Organs from other species were obtained immediately after death, then frozen, and maintained at -20 C or -70 C until extracted. Samples consisted of whole organs in the case of small animals (Wistar strain rats, Swiss white mice and the frog, *Rana pipiens*) and 10 or more portions of 10-20 g each removed from representative areas of the larger animal organs (human and bovine). All samples were first ground to a uniform paste by passage through a household type meat grinder (large animals) or a Potter-Elvehjem homogenizer (small animals). Three samples (100-500 mg) were taken for moisture determination by drying them to constant weight over potassium hydroxide pellets, and one or more additional aliquots (1-10 g) were taken for lipid extraction. The remainder of each homogenate was preserved at -20 C or -70 C in plastic bottles.

Lipid Extraction

In all cases, extraction was accomplished after flushing with nitrogen by homogenization in a Waring blender for 5 min first with 20 vol of chloroform-methanol, 2:1, containing 1 mg of butylated hydroxy toluene (BHT) per liter of solvent. The suspension was filtered under slight vacuum through a medium porosity sintered glass filter under a nitrogen atmosphere. The residue was reextracted three times (3 min each) with 10 vol each of chloroform-methanol, 2:1, chloroform-methanol, 1:2, and finally chloroform-methanol, 7:1 saturated with ammonium hydroxide (28% by weight, freshly prepared) (6). The extracts were pooled and the solvent removed under vacuum, care being taken not to allow the temperature in the flask to rise above 15 C. Methanol and water were removed by repeated additions of chloroform and evaporation to small volume. At no time were the solids taken to complete dryness (7,8).

Following solvent evaporation, lipid was transferred to a Sephadex column in chloroform-methanol, 19:1, saturated with water and the lipids separated from water soluble nonlipid contaminants as previously described (9), except that the acetone wash of Sephadex was eliminated, the bed being washed instead by passing each of the eluting solvents through the bed twice in the same sequence used after sample application. The lipid in fraction 1 from Sephadex was dissolved in a known volume of chloroform-methanol, 9:1. Volumes above 3 ml were prepared in graduated cylinders. Solutions

[1]Permanent address: Department of Neurology, Presbyterian-St. Luke's Hospital, Chicago, Ill.

of smaller volume were prepared in graduated, glass-stoppered 1 ml or 3 ml tubes with graduations to 0.02 ml (Owens-Illinois, Kimble Products Cat. No. 46365, Toledo, Ohio). The total weight of lipid was determined by weighing an aliquot of the solution on a microbalance (8). A 5-6 mg aluminum pan was heated for 3 min at 60-80 C on a melting point block, cooled over potassium hydroxide pellets for 2 min in a desiccator and transferred to a Cahn microbalance for zero adjustment. An aliquot (10-200 μl) of solution was then transferred to the pan which was heated and cooled exactly as before and then weighed to ± 0.1 μg. Heating and cooling periods were carefully timed with a stopwatch and reproduced to within 1 sec.

Preparation of TLC Adsorbent

Silica Gel H (Merck No. 7736, 180 g) was mixed with 20 g of magnesium silicate (Britesorb chromatographic grade; Chemical Specialities Div., Amerace Corp., Tenafly, N.J.) and placed in a wide-mouth screw-cap jar of 2 liter capacity along with 80-90 porcelain grinding balls 0.5 in. in diameter (total weight about 350 g) and heated at 120-150 C for 6 hr. Immediately upon removal from the oven, the bottle was tightly capped and cooled. The adsorbent was then placed on a ball mill and ground for about 2 hr at relatively slow speed.

Preparation of TLC Plates

Glass plates (20 x 20 cm) were first washed in detergent, rinsed with distilled water, air dried and then, immediately before spreading, washed with a stream of chloroform from a plastic wash bottle and air dried. A slurry of adsorbent (20 g) in about 65 ml of 0.01 M potassium hydroxide was spread (Desaga 0.25 mm fixed distance spreader) over the surface of five clean glass plates.

After spreading, plates were stored in air tight chambers until used. Immediately before use, plates were heated at 100-120 C for 30 min and transferred to a humidity controlled chamber (constructed of clear plastic supported on a metal frame) where they were cooled and spotted in a nitrogen atmosphere. The water content of nitrogen inside the chamber was maintained at the desired level by mixing the required amount of dry nitrogen with nitrogen saturated with water by bubbling through a water tower heated electrically at the base. Relative humidity, usually below 60% (range 40-70%) was read from a small meter placed inside the spotting chamber.

Spotting and Chromatographic Development

The TLC chamber (10 3/4 x 2 3/4 in. x 10 1/2 in. high) was prepared for chromatography at the time that heat activation of the plate was begun. The chamber was lined on all sides with Whatman 3 MM paper, about 200 ml of the desired solvent was added, and the liner was saturated with solvent by tilting the covered chamber first to one side and then the other. Sample (200-2000 μg) was spotted from a microsyringe (10-50 μl capacity) as an overlapping row of small spots forming a rectangle about 1 mm wide and 10 mm long. The plate was then transferred to a clear plastic box (only slightly larger than the TLC plate) to minimize gain or loss of water, carried to the TLC chamber, and placed in the chamber as rapidly as possible to avoid extensive loss of solvent vapor from the chamber. After development in the first dimension, the plate was dried for 10 min in a TLC chamber flushed with dry nitrogen and then placed in the second developing solvent. The completed chromatogram was air-dried and sprayed evenly (moderately fine spray) with a mixture of 3 vol of 37% formaldehyde solution plus 97 vol of 98% sulfuric acid to the first appearance of wetness of the adsorbent. The chromatogram was transferred to a forced-draft oven with a glass observation panel in the door and heated at 180 C for 30 min. Color changes were observed during the first few minutes. Almost immediately cholesterol gave a reddish spot that turned black; glycolipids, after a short time, gave purple spots which then turned black, whereas phospholipids charred directly to black spots.

TLC Solvent Systems

Three systems were routinely employed for separation of the polar lipids (phospholipids and glycolipids). These were two-dimensional development with chloroform-methanol-28% aqueous ammonia, 65:25:5, followed by chloroform-acetone-methanol-acetic acid-water, 3:4:1:1:0.5, the same solvents employed in the proportions 65:35:5 and 5:2:1:1:0.5, respectively (10), and chloroform-methanol-water, 65:25:4, followed by 1-butanol-acetic acid-water, 3:1:1 (10).

Column Chromatography and Characterization of Lipids

DEAE cellulose column chromatography (6,8) followed by two-dimensional TLC were employed to determine the extent of spot overlap of components separated by TLC only. Lipid classes were characterized by spray reagents (ninhydrin and colors produced during

TABLE I

Kidney Phospholipids of Various Species[a]

Species	Rat							Human		Bovine	Mouse	Frog
Sample No.	1	2	3	4	5	6	7	8[d]	9[e]	10	11	12
No. of Animals	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(12)	(9)
H_2O, %	75.8	75.6	76.0	75.0	74.5	74.6	75.0	78.1	78.0	70.34	74.98	82.5
Lipid[b], %	4.69	4.75	4.45	4.02	3.74	3.37	3.45	2.12	5.26	3.26	5.78	1.84
Lipid P[c]	2.50	2.64	2.63	3.05	2.63	3.03	3.05	2.43	1.09	1.25	1.43	1.89
No. of determinations[f]	4	4	4	4	3	4	4	4	4	8	8	8
Phosphatidyl choline	35.0 ±0.2	34.4 ±0.6	35.2 ±0.8	32.2 ±0.5	34.8 ±0.6	34.8 ±0.3	33.6 ±0.5	31.6 ±0.1	34.6 ±0.2	32.4 ±0.2	35.6 ±0.4	34.3 ±0.5
Phosphatidyl ethanolamine	27.3 ±0.2	27.8 ±0.2	27.9 ±0.5	26.5 ±1.0	27.8 ±0.3	26.3 ±0.3	26.3 ±0.1	26.5 ±0.4	28.2 ±0.1	28.6 ±0.2	26.4 ±0.6	30.0 ±0.5
Phosphatidyl serine	7.4 ±0.1	7.7 ±0.2	6.9 ±0.2	6.3 ±0.1	7.5 ±0.3	7.3 ±0.1	7.7 ±0.3	6.3 ±0.3	6.4 ±0.2	7.5 ±0.3	7.0 ±0.2	6.0 ±0.1
Phosphatidyl inositol	6.0 ±0.1	6.0 ±0.2	5.9 ±0.2	5.7 ±0.2	5.6 ±0.1	6.0 ±0.1	5.9 ±0.1	5.4 ±0.1	5.6 ±0.2	7.2 ±0.3	5.7 ±0.1	5.6 ±0.0
Phosphatidic acid	0.3 ±0.1	0.2 ±0.1	0.3 ±0.2	Trace	0.2 ±0.1	0.2 ±0.1	0.6 ±0.2	Trace	0.4 ±0.2	0.2 ±0.1	0.2 ±0.1	0.3 ±0.1
Diphosphatidyl glycerol	6.6 ±0.1	7.2 ±0.2	6.5 ±0.1	6.4 ±0.3	6.7 ±0.3	5.9 ±0.4	6.3 ±0.2	4.4 ±0.2	4.0 ±0.2	6.5 ±0.1	7.3 ±0.3	4.8 ±0.2
Phosphatidyl glycerol	0.2 ±0.1	0.5 ±0.1	0.3 ±0.1	0.3 ±0.1	0.3 ±0.1	0.1 ±0.05	0.2 ±0.05	0.3 ±0.1	0.8 ±0.2	0.4 ±0.2	0.5 ±0.2	0.3 ±0.1
Lysobisphosphatidic acid	0.1 ±0.1	0.2 ±0.1	0.07 ±0.05	0.1 ±0.05	0.2 ±0.1	Trace	0.1 ±0.1	0.2 ±0.1	Trace	0.2 ±0.1	0.2 ±0.1	0.4 ±0.2
Lysophosphatidyl choline	1.0 ±0.3	0.9 ±0.2	0.8 ±0.2	0.8 ±0.1	1.0 ±0.2	1.0	1.1 ±0.2	3.6 ±0.1	2.1 ±0.2	0.7 ±0.2	0.9 ±0.2	0.2 ±0.2
Lysophosphatidyl ethanolamine	ND[g]	0.4 ±0.1	0.2 ±0.1	0.1 ±0.05	0.4 ±0.2	Trace	Trace	3.8 ±0.0	2.1 ±0.2	0.3 ±0.2	ND	ND
Sphingomyelin	12.4 ±0.1	13.3 ±0.4	12.3 ±0.3	12.5 ±0.3	12.3 ±0.1	11.3 ±0.2	10.8 ±0.2	12.9 ±0.3	11.1 ±0.3	13.7 ±0.2	10.4 ±0.3	12.2 ±0.5
Sum	96.3	98.6	96.4	90.9	96.8	92.9	92.6	95.0	95.3	97.7	94.2	94.1
P recovery, %	97.9	98.7	97.8	93.0	98.6	94.3	94.0	97.2	98.1	97.8	97.5	97.9
Total unidentified	3.7	1.4	3.6	9.1	3.2	7.1	7.4	5.0	4.7	2.3	5.8	5.9

[a]As percentage of the total lipid phosphorus (in Sephadex fraction 1) ± standard deviation.

[b]As percentage of the fresh weight.

[c]Milligram of phosphorus per 100 mg of lipid.

[d]Obtained 8 hr post-mortem.

[e]Obtained 1 3/4 hr post-mortem.

[f]Replicate TLC determinations of each phospholipid class.

[g]ND, not detected.

TABLE II

Liver Phospholipids of Various Species[a]

Species	Rat									Mouse	Frog	Bovine	Human[e]
Sample No.	1	2	3	4[d]	4[d]	4[d]	5	6	7	8	9	10	11
Analysis No.	1	2	3	4	5	6	7	8	9	10	11	12	13
No. Animals	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(7)	(3)	(1)	(1)
H_2O, %	77.75	78.50	77.72	70.76	[f]	[f]	71.04	72.69	70.62	70.69	70.90	73.60	67.3
Lipids[b], %	4.97	4.67	4.96	4.06	[f]	[f]	3.90	4.06	4.20	4.33	4.62	4.20	14.7
Lipid P[c]	2.58	2.70	2.58	2.87	[f]	[f]	2.80	2.91	2.83	1.85	1.00	1.91	0.90
No. determinations	8	8	8	4	4	4	4	4	4	7	8	8	8
Phosphatidyl choline	50.4 ±0.2	50.0 ±0.3	47.4 ±0.7	46.7 ±0.1	47.1 ±0.3	47.4 ±0.4	47.3 ±0.1	47.9 ±0.3	49.5 ±0.8	45.6 ±1.0	46.5 ±0.5	52.9 ±0.6	43.6 ±0.3
Phosphatidyl ethanolamine	23.0 ±0.5	24.7 ±0.6	22.1 ±0.3	22.7 ±0.1	23.4 ±0.1	23.0 ±0.6	24.5 ±0.2	24.0 ±0.6	24.9 ±0.6	25.1 ±0.6	25.6 ±0.4	24.4 ±0.4	27.9 ±0.2
Phosphatidyl serine	3.5 ±0.2	2.8 ±0.0	3.4 ±0.1	3.2 ±0.1	3.4 ±0.0	3.2 ±0.0	3.6 ±0.1	3.6 ±0.0	3.8 ±0.2	3.9 ±0.1	3.9 ±0.1	4.1 ±0.2	3.1 ±0.2
Phosphatidyl inositol	7.8 ±0.2	7.9 ±0.0	7.9 ±0.1	8.0 ±0.1	8.5 ±0.1	8.7 ±0.2	8.9 ±0.0	8.2 ±0.1	8.8 ±0.2	7.7 ±0.1	7.3 ±0.1	8.3 ±0.2	8.6 ±0.2
Phosphatidic acid	0.6 ±0.2	0.2 ±0.1	0.3 ±0.1	0.1 ±0.05	Trace	0.2 ±0.1	0.2 ±0.1	0.2 ±0.1	0.4 ±0.2	0.1 ±0.0	0.2 ±0.1	0.4 ±0.2	0.7 ±0.1
Diphosphatidyl glycerol	3.6 ±0.2	4.3 ±0.3	5.2 ±0.2	4.7 ±0.3	4.7 ±0.2	4.7 ±0.2	4.7 ±0.2	4.3 ±0.0	4.7 ±0.2	4.7 ±0.3	2.0 ±0.2	2.7	3.7 ±0.2
Phosphatidyl glycerol	0.3 ±0.1	0.1 ±0.05	0.2 ±0.1	0.2 ±0.1	0.5 ±0.2	Trace	0.3 ±0.1	0.3 ±0.1	Trace	0.1 ±0.1	0.2 ±0.1	0.2 ±0.1	ND
Lysobisphosphatidic acid	ND	0.2 ±0.1	0.1 ±0.05	ND	0.3 ±0.2	Trace	0.4 ±0.2	Trace	0.2 ±0.1	0.1 ±0.1	0.4 ±0.3	0.4 ±0.2	1.0 ±0.3
Lysophosphatidyl choline	0.9 ±0.2	0.6 ±0.1	0.5 ±0.1	0.5 ±0.2	0.9 ±0.2	0.7 ±0.2	1.3 ±0.8	0.8 ±0.1	0.8 ±0.2	0.7 ±0.1	0.8 ±0.3	0.9 ±0.3	1.3 ±0.3
Lysophosphatidyl ethanolamine	ND	ND	0.4 ±0.2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sphingomyeline	4.1 ±0.1	4.3 ±0.1	4.4 ±0.2	3.9 ±0.3	3.8 ±0.4	4.2 ±0.4	4.3 ±0.2	3.9 ±0.1	4.2 ±0.1	4.7 ±0.3	5.1 ±0.7	4.3 ±0.3	4.6 ±0.3
Sum	94.2	95.1	91.9	90.0	92.6	92.1	95.5	93.2	97.3	92.7	92.0	98.6	94.5
P recovery, %	97.6	99.8	97.4	97.6	98.9	99.2	97.6	96.4	100.4	97.4	98.2	99.4	98.8
Total unidentified	5.8	4.9	8.1	10.0	7.4	7.9	4.4	6.8	2.7	7.3	8.0	1.4	5.5

[a,b,c]As for Table I.

[d]Sample 4 was analyzed on three separate occasions at two week intervals.

[e]Obtained 1 3/4 hr post-mortem.

[f]Values shown in first analysis.

TABLE III

Spleen Phospholipids of Various Species[a]

Species	Rat				Mouse	Human[d]	Bovine
Sample No.	1	2	3	4	5	6	7
No. Animals	(3)	(1)	(1)	(1)	(12)	(1)	(1)
H_2O, %	76.23	77.64	77.71	75.38	78.61	76.40	77.0
Lipid[b], %	2.76	1.40	2.16	2.18	2.27	3.99	5.06[e]
Lipid P[c]	2.31	2.65	2.57	2.68	1.88	1.98	0.50
No. determinations	7	4	4	4	8	8	8
Phosphatidyl choline	41.9 ±0.4	41.5 ±0.3	41.9 ±0.2	42.3 ±1.5	41.5 ±1.4	41.2 ±0.2	36.0
Phosphatidyl ethanolamine	23.9 ±0.9	24.6 ±0.2	23.9 ±0.1	23.7 ±1.1	24.9 ±0.6	24.5 ±0.2	24.5 ±0.2
Phosphatidyl serine	8.3 ±0.2	7.4 ±0.3	8.4 ±0.1	7.9 ±0.2	7.9 ±0.1	8.3 ±0.3	12.0 ±0.2
Phosphatidyl inositol	5.0 ±0.1	5.8 ±0.3	5.6 ±0.1	5.7 ±0.1	5.8 ±0.2	4.4 ±0.2	4.3 ±0.2
Phosphatidic acid	0.4 ±0.2	0.1 ±0.1	0.7 ±0.2	0.6 ±0.2	0.2 ±0.1	0.2 ±0.1	0.6 ±0.2
Diphosphatidyl glycerol	1.7 ±0.2	2.5 ±0.1	2.1 ±0.2	1.9 ±0.2	2.0 ±0.2	1.0 ±0.1	0.3 ±0.1
Phosphatidyl glycerol	2.2 ±0.3	0.1 ±0.1	0.5 ±0.2	0.3 ±0.1	0.3 ±0.2	0.3 ±0.1	ND
Lysobisphosphatidic acid	0.5 ±0.2	Trace	0.7 ±0.3	0.5 ±0.0	0.7 ±0.2	0.3 ±0.1	ND
Lysophosphatidyl choline	1.2 ±0.1	1.2 ±0.2	1.5 ±0.2	1.6 ±0.1	1.5 ±0.2	1.5 ±0.2	0.8 ±0.4
Lysophosphatidyl ethanolamine	ND	ND	ND	ND	ND	ND	0.3 ±0.1
Sphingomyelin	7.3 ±0.2	6.4 ±0.3	6.4 ±0.2	6.4 ±0.2	6.6 ±0.3	12.8 ±0.4	14.5 ±0.3
Sum	92.4	89.6	91.7	90.9	91.4	94.5	93.3
P recovery, %	97.5	95.9	96.6	97.6	97.1	98.9	98.3
Total unidentified	7.6	10.4	8.3	9.1	8.6	5.5	6.7

[a,b,c]As for Table I.

[d]Obtained 1 3/4 hr post-mortem.

[e]More triglyceride was present in the sample.

charring), phosphorus analysis (10), ester group determination (11), chromatographic migration, identification of hydrolysis products (6) and infrared spectrophotometry (6).

Determination of Phosphorus After TLC

The procedure previously described (10) and a more sensitive modification (reduction of reagent volumes to one third of those previously described) were used for determination of phosphorus and hence the molar amounts of phospholipids.

RESULTS

Evaluation of Analytical Data

The phospholipid class distribution of kidney (Table I), liver (Table II) and spleen (Table III) can be altered by postmortem enzymatic degradation. We found that values for rat and bovine kidney did not change appreciably when organs were allowed to stand at 23 C for 5-8 hr. The values in Table I are thus not affected significantly by such changes. In contrast, liver phospholipids were found to undergo degradation with some alteration of phospholipid class distribution within 1 hr (12). Since livers from species other than man were obtained immediately, only the values for human liver (Table II) are in some error from postmortem degradation. Upon standing at 23 C, spleen phospholipids were degraded less rapidly than liver but more rapidly than kidney. Thus, even the values for human spleen (Table III) were not appreciably altered by postmortem changes.

Alteration of lipid class distribution was observed to occur when lipid extracts were evaporated completely to dryness and when solutions without an antioxidant were allowed to stand (-20 C or -70 C). Such alterations were prevented by use of the solvent replacement

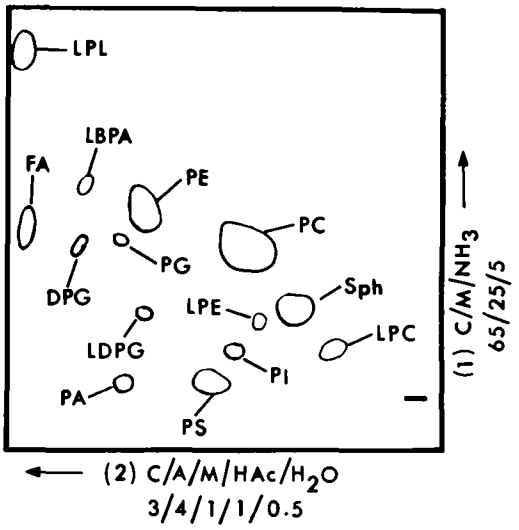

FIG. 1. Map of spots obtained by TLC (600-800 μg of sample applied at the lower right side with chloroform-methanol-28% aqueous ammonia, 65:25:5, followed by chloroform-acetone-methanol-acetic acid-water, 3:4:1:1:0.5. Excellent resolution of phospholipid classes is obtained with this system. Note by comparison with Fig. 2, for which the same solvents in different proportions were used for development, that increase of the amount of methanol or acetone causes a greater change in the migration of acidic phospholipids as compared to the nonacidic phospholipids. Abbreviations: LPL, less polar lipids (cholesterol, triglyceride, etc.); LBPA, lysobisphosphatidic acid; FA, free fatty acid; DPG, diphosphatidyl glycerol; PG, phosphatidyl glycerol; PE, phosphatidyl ethanolamine; LDPG, lysodiphosphatidyl glycerol sometimes seen in extracts of organs allowed to stand for several hours before extraction; PC, phosphatidyl choline; PA, phosphatidic acid; PS, phosphatidyl serine; PI, phosphatidyl inositol; LPE, lysophosphatidyl ethanolamine; Sph, sphingomyelin; LPC, lysophosphatidyl choline.

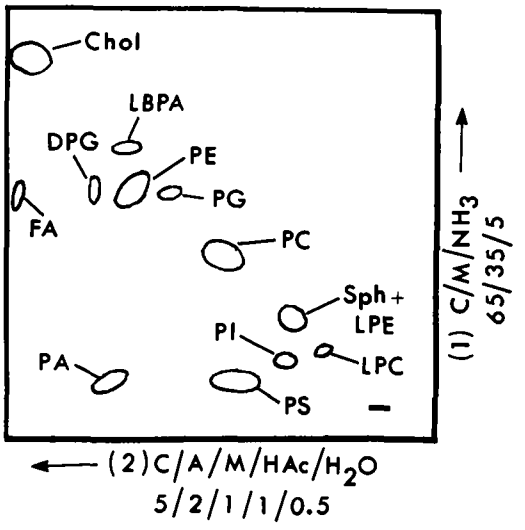

FIG. 2. Map of spots obtained by TLC with the solvents used for Figure 1 but in the proportions 65:35:5 and 5:2:1:1:0.5 for the first and second dimensions, respectively. See Figure 1 for abbreviations and comments.

procedure and weighing of an aliquot of a solution (described under Methods) and by addition of an antioxidant. BHT proved to be a convenient antioxidant since it is effective in small amount and most of it is lost during solvent evaporation, thus making careful addition of an exact amount unnecessary.

The accuracy of quantitative TLC determinations was judged in part by demonstration that each spot was produced only from the components indicated in Tables I-III. This was accomplished by two-dimensional TLC with 3 different solvent pairs, with which relative migrations of the phospholipids are different, and by ion exchange cellulose column chromatography which separates lipid classes in a characteristic manner that is different from TLC. Spot identifications in Figures 1-3 were supported by all TLC and column chromatographic findings. Each spot showed the characteristic chromatographic migration of the substance designated and phosphorus determinations showed the spots after TLC with different systems to contain the expected amount of phosphorus. It was thus judged that spots shown in Figures 1-3 were produced from the lipid classes shown and that the values in Tables I-III are for individual phospholipids classes. Reproducibility which also contributes to overall accuracy was demonstrated by satisfactory values for standard deviations.

Phosphorus recoveries with pure standards were 100% ± 0.5-1.0%. The lower recoveries obtained with organ extracts (Tables I-III) were traced to the presence of uncharacterized substances. Some of these substances occurred at very low level and thus could not be observed by TLC alone, although their presence was disclosed by TLC of DEAE column fractions. Most of the uncharacterized phosphorus-containing substances were found in the last (acidic) fraction eluted with chloroform-methanol 2:1 made 0.1 N in potassium acetate and containing 20 ml of 28% aqueous ammonia per liter. Fortunately, these uncharacterized components were separable by two-dimensional TLC from the lipid classes shown in Tables I-III. Some areas of chromatograms, although devoid of spots, were taken for analysis. Other areas devoid of spots and not analyzed also contained phosphorus and thus, total phosphorus recovery was less than 100%.

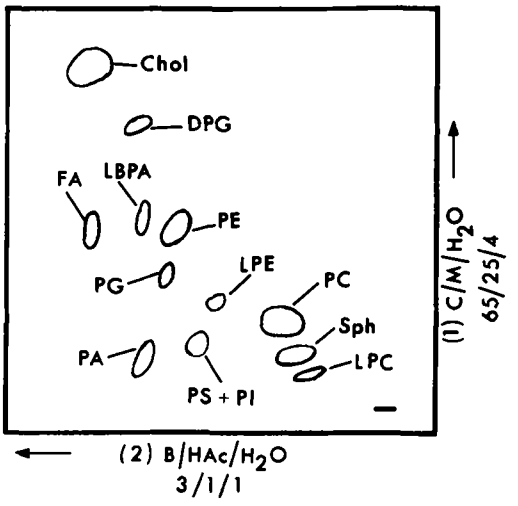

FIG. 3. Map of spots obtained by TLC with chloroform-methanol-water, 65:25:4, followed by 1-butanol-acetic acid-water, 3:1:1. See Figure 1 for abbreviations and comments.

Variations in Phospholipid Class Distribution Within One Species and Among Different Vertebrates

Rat kidney was investigated for variations within a species (Table I). The data for different animals are similar, but some biological variability is evident. The variability within one species is of the same order as that observed for different species of vertebrates.

Some biological variability is apparent (Table II) for rat liver phospholipid class distribution which is clearly not methodological error as shown by the three separate analyses of one sample (4-6, Table II). Differences among the various vertebrate species are similar to those obtained for rat livers.

The data for rat spleens (analyses 1-4, Table III) indicate little variability within one species. The values for the other species fall into two groups, i.e., those with sphingomyelin levels of about 6.7% and 13.6%, respectively, although the values for phospholipids other than diphosphatidyl glycerol are closely similar (see Discussion).

DISCUSSION

Factors affecting phospholipid class distributions were evaluated in the present study. These included alteration by postmortem enzymatic degradation, methods of preparing lipid extracts for analysis, accuracy and reproducibility of analytical methods and determination of intra- as well as interspecies variability. Separation by two-dimensional TLC followed by determination of phosphorus content of spots is a relatively rapid procedure which provides accurate values. Satisfactory separations are not obtained by one-dimensional TLC, and DEAE column chromatography followed by TLC is more time-consuming. The latter procedure is, however, excellent for demonstration of the number of lipid classes represented by each spot obtained by direct two-dimensional TLC of lipid extracts.

The kidney, like lung, heart and skeletal muscle in particular, is an organ that performs the same function in various vertebrate species and is also relatively stable to change of its phospholipid composition by postmortem enzymatic degradation. It is thus an organ with which species variations in phospholipid class distribution can be determined with relative ease. The close similarity of kidney phospholipid values for all species corroborates the conclusion that, among vertebrates, neither the relative proportions of different membranes of organs nor the phospholipid composition of the individual types of membrane varies appreciably (1-5). Data for liver lead to similar conclusions. Spleen is an organ that is not present in some species (e.g., frog). Also, hematopoiesis takes place in the spleen in some species. The differences in sphingomyelin values for the smaller and larger species of animals may be explained by this difference. The level of diphosphatidyl glycerol is much lower in human and bovine spleens. In most organs, diphosphatidyl glycerol is present only in mitochondria which contain little or no sphingomyelin (13). A lower level of diphosphatidyl glycerol and a higher level of sphingomyelin thus indicate a small contribution of mitochondrial lipid to the phospholipid composition of human and bovine spleens.

ACKNOWLEDGMENTS

Supported in part by U.S. Public Health Service Grants NB06237 and NB06113.

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[Received January 22, 1969]

Species Variations in Phospholipid Class Distribution of Organs: II. Heart and Skeletal Muscle

GERALD SIMON, Department of Neurology, Presbyterian-St. Luke's Hospital, Chicago, Illinois 60612 and **GEORGE ROUSER**, Section of Lipid Research, Division of Neurosciences, City of Hope Medical Center, Duarte, California 91010

ABSTRACT

Total lipid, lipid phosphorus and phospholipid class distribution were determined for heart and skeletal muscles from five vertebrates (human, bovine, rat, mouse and frog) and skeletal muscles from four invertebrates (lobster, abalone, scallop and sea urchin). The precision of the analytical method [separation by two-dimensional thin layer chromatography (TLC) and phosphorus analysis of spots] was demonstrated by small values for standard deviations. Accuracy of spot identification and analytical values was insured by comparison with results obtained by TLC of triethylaminoethyl cellulose column chromatographic fractions. Values for total lipid, total phospholipid and phospholipid class distribution of heart and skeletal muscles from the five vertebrate species showed essentially the same variability observed for the same organ from different animals of one species (rat). The data indicate that, among vertebrates, there is little or no variability for phospholipid class distribution in muscle membranes in agreement with data for other organs and subcellular particulates presented in other reports. Invertebrate skeletal muscles were found to differ qualitatively and/or quantitatively from those of vertebrates. In one species (sea urchin), ceramide phosphorylethanolamine was the only sphingolipid, sphingomyelin characteristic of vertebrates being absent. In two species (abalone and scallop) ceramide aminoethylphosphonate was present and sphingomyelin was absent. In one (lobster), sphingomyelin was the only sphingolipid. Quantitatively, higher levels of sphingolipid and phosphatidyl serine and lower levels of phosphatidyl inositol were found in invertebrate skeletal muscle. The significance of the data is discussed in relation to subcellular particulate lipid class composition.

INTRODUCTION

This report is one of a series in which data are presented for species variation of animal cell membrane phospholipid class distribution as disclosed by analysis of whole organs and subcellular particulates. Heart and skeletal muscle are considered in this report. Data for other organs and for highly purified subcellular particulates are presented in other reports (1-5).

MATERIALS AND METHODS

Beef heart was obtained 20 min postmortem, frozen and maintained at -20 C until extracted. A normal human heart was obtained 1 hr and 45 min postmortem, frozen and maintained at -20 C until extracted. Hearts from rats (Wistar strain), mice (Swiss white) and frogs (*Rana pipiens*) were obtained immediately after decapitation, weighed and extracted.

Skeletal muscle was obtained from the legs of mouse, rat, frog and human and from bovine neck. Invertebrate muscle was obtained from animals caught by the investigators off the Channel Islands near the coast of Southern California. As soon as a specimen was taken, it was immediately brought to the surface, the muscle removed, placed in a plastic bag, and frozen on dry ice. No more than 3 min elapsed between the time any specimen was taken and the muscle was placed on dry ice. Each species was caught in the same general locality. Thus, all Large Rock Scallops (*Hinnites giganteum*) were collected off the north coast of Santa Cruz Island, all Pink Abalone (*Haliotis corrugata*) were caught off the south coast of Santa Catalina Island, all Giant Red Sea Urchins (*Strongylocentrotus franciscanus*) were caught off the west coast of Santa Catalina Island, and all Spiny Lobster (*Panulirus interruptus*) were caught off the west coast of San Clemente Island. Muscle from eight individuals was pooled prior to extraction of the lipids from each species.

Lipid Extraction and Analysis

Heart muscle samples were first cut into small pieces and then homogenized in a Waring blender with chloroform-methanol. Skeletal muscle was first frozen in liquid nitrogen, powdered in a cold piston-cylinder device, and then homogenized in the blender with extracting solvent. In all cases, extraction, Sephadex column chromatography and quantitative TLC were carried out as described in an accompanying report (1).

TEAE Column Chromatography

Selectacel TEAE standard grade (Brown Co., Berlin, New Hampshire) was washed (0.01 N potassium hydroxide and 1 N hydrochloric acid) and dried as previously described for DEAE cellulose (6). The washed, dry preparation (15 g) was left in glacial acetic acid overnight and packed into a 2.5 (i.d.) x 30 cm chromatographic tube equipped with a 1 liter solvent reservoir and Teflon stopcock. Columns were packed by first placing a glass wool plug in the tube partially filled with solvent, removing air bubbles, then adding a small portion of TEAE slurry. Excess solvent was allowed to fall to the top of the bed which was then compressed manually with the aid of a large bore glass rod and the uppermost part of the bed stirred. This process was repeated four to five times to give a final height of 20 ± 2 cm. Acetic acid was washed out with 6 column volumes of methanol. The bed was then washed with 3 column volumes of 0.01 N potassium hydroxide in methanol, 6 column volumes of methanol, 3 column volumes of chloroform-methanol, 1:1 and 3 column volumes of chloroform.

Each newly packed column was tested by applying about 1 mg of azulene in 3-5 ml of chloroform (7). Solvent was collected in a graduated cylinder from the time the blue hydrocarbon solution was applied. The volume collected to the first appearance of azulene (about 75 ml for a 2.5 x 20 cm bed) was recorded as the column volume and any uneven portions of the bed were easily observed as the narrow band of azulene moved down the bed. If the bed was observed to be packed in an uneven manner, it was extruded and repacked in chloroform and retested. Samples (100-200 mg) were applied in the first eluting solvent.

Chloroform, methanol and glacial acetic acid were redistilled before use. Aqueous ammonia (28% by weight) was prepared by passing gaseous ammonia into ice-cold distilled water in a Teflon bottle. The ammonia was discarded after several days when nonvolatile solids appeared.

Elution of the column (7) was as follows: Fraction 1, chloroform (7 column volumes) for neutral (less polar) lipids; Fraction 2, chloroform-methanol, 9:1 (5 column volumes) for phosphatidyl choline, lysophosphatidylcholine and sphingomyelin; Fraction 3, chloroform-methanol, 2:1 (7 column volumes) for ceramide polyhexosides; Fraction 4, chloroform-methanol, 2:1, containing 1% (v/v) glacial acetic acid (8 column volumes) for phosphatidyl ethanolamine, lysophosphatidyl ethanolamine, ceramide phosphorylethanolamine, ceramide aminoethylphosphonate and free fatty acid; Fraction 5, glacial acetic acid (5 column volumes) for phosphatidyl serine; Fraction 6, a methanol wash (4 column volumes) to remove acetic acid (added to Fraction 5 for evaporation); Fraction 7, chloroform-methanol, 2:1 made 0.1 N in potassium acetate and containing 20 ml of 28% aqueous ammonia per liter (8 column volumes) for phosphatidic acid, phosphatidyl inositol, diphosphatidyl glycerol, phosphatidyl glycerol, lysobisphosphatidic acid and traces of other uncharacterized acidic lipids; and Fraction 8, a methanol wash (4 column volumes) to clear the column (added to Fraction 7 for evaporation). Salt in the last fraction was removed by Sephadex column chromatography (1).

Phospholipid Identification and Quantitation

The lipid classes of TEAE column fractions were separated by two-dimensional TLC and visualized by charring (1). All lipid classes were identified on the basis of their characteristic chromatographic properties (TEAE column chromatography and two dimensional TLC) and reactivity to spray reagents (ninhydrin and formaldehyde-sulfuric acid). Ceramide phosphorylethanolamine was identified by comparison with an authentic sample provided by T. Hori. In addition, ceramide aminoethylphosphonate was identified on the basis of its hydrolysis products (6,8).

Isolation of Ceramide Aminoethylphosphonate from Scallop Skeletal Muscle

The lipid eluted in TEAE column Fraction 4 was spotted onto TLC plates which were developed with chloroform-acetone-methanol-acetic acid-water, 5:2:1:1:0.5. The lipid bands were located by spraying with water, scraped from the plate, and lipid eluted quantitatively from the wet adsorbent with chloroform-methanol, 2:1. The solvent was removed under a stream of nitrogen, the lipid (dissolved in chloroform-methanol, 19:1, saturated with water) separated from silica gel and salt by application to a 1 x 10 cm Sephadex column and elution with chloroform-methanol, 19:1 saturated with water. Solvent was removed under vacuum and the lipid stored in chloroform-methanol, 9:1 at -20 C.

Degradation of Ceramide Aminoethylphosphonate

Lipid was placed in a Teflon-lined screw-capped tube, hydrolyzed (8), and lipid extracted into chloroform. The hydrolysis products were examined by paper chromatography and TLC (6,8). Sphingosine, dihydro-

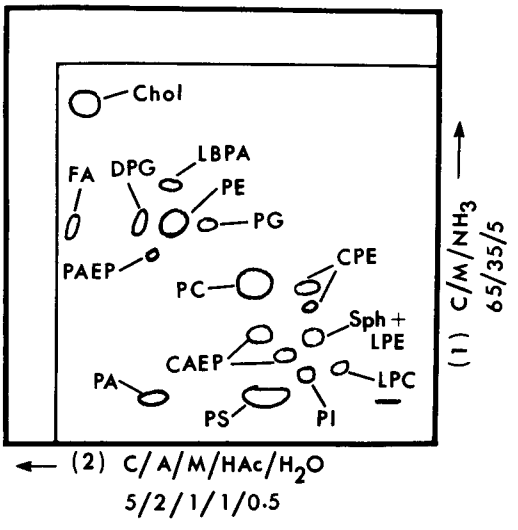

FIG. 1. Diagram of the positions of the different lipid classes after development first with chloroform-methanol 28% aqueous ammonia 65:35:5 and then chloroform-acetone-methanol-acetic acid-water 5:2:1:1:0.5. Abbreviations: Chol, cholesterol; LBPA, lysobisphosphatidic acid: FA, fatty acid; DPG, diphosphatidyl glycerol (cardiolipin); PAEP, phosphatidyl 2-aminoethylphosphonate; PE, phosphatidyl ethanolamine; PC, phosphatidyl choline; CPE, ceramide phosphorylethanolamine; CAEP, ceramide aminoethylphosphonate; Sph, sphingomyelin; LPE, lysophosphatidyl ethanolamine; LPC, lysophosphatidyl choline; PI, phosphatidyl inositol; PS, phosphatidyl serine; PA, phosphatidic acid.

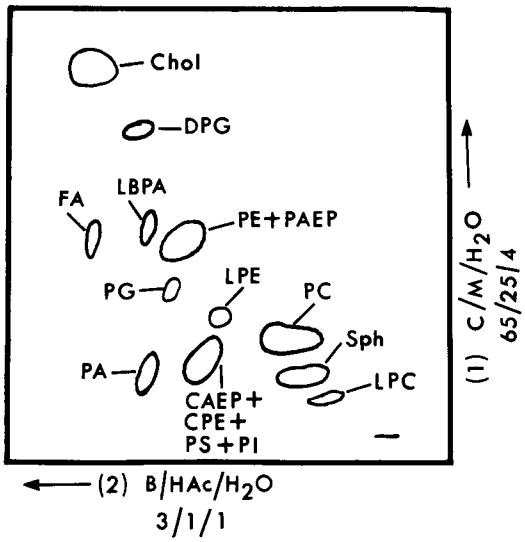

FIG. 2. Diagram of the positions of the different lipid classes after development first with chloroform-methanol-water 65:25:4 and then 1-butanol-acetic acid-water 3:1:1. Abbreviations as for Fig. 1. Spots from many lipid classes overlap with this system, but lysophosphatidyl ethanolamine is separated from the other phospholipids and the different relative order of migration of lipid classes with the system is useful for identification of lipid classes. The position of lysophosphatidyl ethanolamine relative to the other phospholipids is somewhat variable. It may migrate somewhat above or below the position shown.

sphingosine and phytosphingosine were used as standards.

Isolation and Characterization of the Phosphonic Acid Analogue of Phosphatidyl Ethanolamine

In scallop skeletal muscle, a small ninhydrin-positive spot was detected just below the usual phosphatidyl ethanolamine spot. The substance was eluted from TEAE in Fraction 4, the ethanolamine phosphoglyceride fraction. It was separated from phosphatidyl ethanolamine by TLC with chloroform-methanol-28% aqueous ammonia, 65:35:5 as solvent, located by spraying with water, eluted with chloroform-methanol 2:1 saturated with water, freed of nonlipid contaminants by Sephadex column chromatography, hydrolyzed and hydrolysis products examined as described for ceramide aminoethylphosphonate (6,8).

RESULTS

Separation, Identification and Determination of Lipid Classes

The positions of the different lipid classes after TLC with the two systems used routinely for analysis (Fig 1-3) were quite reproducible. When both two-dimensional systems are used, separation (without spot overlap) of the lipid classes shown in Figures 1 and 2 is possible. Thus, each lipid class shown can be determined separately. TLC of TEAE column fractions was used to disclose overlap of spots seen by TLC without column chromatography. These examinations demonstrated that the spot identifications in Figures 1 and 2 are correct, and that there were no other lipid classes in each spot.

TEAE cellulose column chromatography is particularly useful for demonstrating spot overlap by TLC because phospholipids are eluted on the basis of differences in the types of ionic groups, whereas other factors influence TLC separations. With TEAE, all choline lipids are eluted in Fraction 2, ethanolamine and 2-aminoethylphosphonic acid lipids in Fraction 4, serine lipids in Fraction 5, and acidic phospholipids (phosphate as the only ionic group) in Fraction 6, although some Fraction 6 components are eluted in part in Fraction 5. This difficulty was overcome by passage of Fraction 5 through a DEAE column on which Fraction 6 components are retained and thus separated completely from phosphatidyl serine.

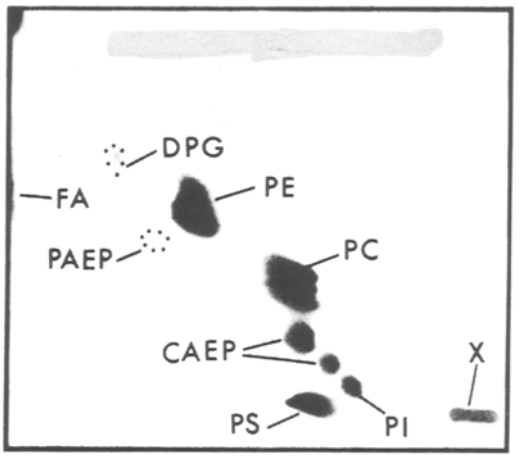

FIG. 3. TLC separation of scallop muscle phospholipids. The chromatogram was developed as described in the legend for Figure 1. Note that ceramide aminoethylphosphonate in this species gives two completely separated spots which were shown to be from the same lipid class by demonstration of sphingosine, fatty acid and 2-aminoethylphosphonic acid as hydrolysis products.

TEAE differs from DEAE in retaining the ethanolamine lipids when eluted with chloroform-methanol, 2:1 (Fraction 3) and thus, with TEAE, use of the latter solvent gives a fraction composed only of ceramide polyhexosides (two or more hexose moieties) when these are present. TEAE Fraction 3 from all muscle samples was devoid of glycolipid in keeping with the failure to find by TLC typical glycolipid spots which, with the formaldehyde-sulfuric acid spray, give a purple color turning to black. TLC of TEAE column Fraction 2 at high (1 mg) load also failed to disclose the presence of even a trace of sphingomyelin in skeletal muscles from abalone, scallop and sea urchin. Similar examination of TEAE Fraction 4 failed to disclose even a trace of ceramide phosphorylethanolamine or ceramide aminoethylphosphonate in lobster skeletal muscle and vertebrate heart and skeletal muscles.

It is noteworthy that the phosphonolipids are separable from the corresponding phosphodiester lipids with solvent mixtures used for Figure 1 but not those used for Figure 2. Also, two or more spots are obtained for different molecular species of ceramide aminoethylphosphonate and ceramide phosphorylethanolamine in some species. It is thus essential for identification of components that each spot be isolated and characterized by the products obtained after hydrolysis.

Species Variability of Heart Phospholipid Composition

The variability of composition within one species is indicated by the values (Table I) for rat and frog hearts. Water content was quite reproducible, although total lipid and total lipid P (mg/100 mg lipid) showed more variability which was related to the variable amount of triglyceride in samples. The values for the rat heart phospholipid classes (as percentage of the total lipid phosphorus) were in all cases very similar. The variability noted was biological and not methodological since the same values were obtained for samples when rerun. This high degree of reproducibility demonstrates the absence of large biological variations and the good reproducibility of the procedures for extraction, Sephadex column chromatography, and quantitative determination of phosphorus after TLC.

The phospholipid values (Table I) for mouse, bovine, human and frog hearts show in general about the same order of variability as that found for different rat hearts. There is a distinctly higher level of sphingomyelin associated with a lower level of diphosphatidyl glycerol in frog heart. This suggests that mitochondria (which contain diphosphatidyl glycerol) are less abundant in frog heart muscle. Total lipid phosphorus recovery from most heart samples was lower than that obtained with pure standards (100% ± 0.5-1.0%) and with skeletal muscle (about 99.5% ± 1.0%, Table II). The lower recovery is related to the presence of uncharacterized phosphorus-containing substances, some of which are not visualized with the char spray. Most of the uncharacterized phospholipid was eluted from TEAE in the last (acidic) fraction in which other minor components not detectable by direct TLC of the mixture were also observed. Chromatographic separation of water soluble compounds released by mild alkaline hydrolysis indicated that some of the minor uncharacterized lipids were lyso compounds related to bisphosphatidic acid and diphosphatidyl glycerol.

Species Variability of Skeletal Muscle Phospholipid Composition

The large variability of values for total lipid and lipid phosphorus was clearly related to large differences in triglyceride content of different samples. The phospholipid class values obtained for skeletal muscle of vertebrates (Table II) are different from those obtained for heart muscle, although like heart muscle, the striking feature of the vertebrate skeletal muscle values is their close similarity. The largest variation was in the diphosphatidyl glycerol (cardiolipin) content.

TABLE I

Heart Muscle Phospholipids[a]

Species	Rat					Mouse	Bovine	Human	Frog		
Analysis no.	1	2	3	4	5	6	7	8	9	10	11
No. animals	3	1	1	1	1	12	1	1	8	2	2
H_2O, %	77.30	77.38	76.66	76.55	77.41	77.80	78.86	78.90	90.9	91.2	90.2
Lipid[b], %	3.62	2.78	2.59	1.69	2.22	3.12	4.53	2.81	1.90	1.85	2.26
Lipid P[c]	2.41	1.48	1.62	1.81	1.79	1.80	1.00	2.37	1.37	1.65	2.16
No. determinations[d]	8	8	8	8	8	8	8	8	8	4	4
Phosphatidyl choline	36.8 ±0.4	36.3 ±0.3	34.4 ±0.1	36.5 ±0.4	36.0 ±0.1	40.6 ±0.2	38.1 ±0.3	40.0 ±0.3	36.5 ±0.2	41.1 ±0.8	41.4 ±0.7
Phosphatidyl ethanolamine	30.8 ±0.3	29.1 ±0.1	29.0 ±0.3	29.9 ±0.0	29.8 ±0.2	28.9 ±0.1	29.7 ±0.3	26.3 ±0.4	29.2 ±0.1	32.7 ±0.4	32.6 ±0.3
Phosphatidyl serine	2.9 ±0.1	3.5 ±0.1	3.0 ±0.1	3.4 ±0.2	3.3 ±0.1	2.8 ±0.1	3.5 ±0.5	2.7 ±0.2	4.7 ±0.2	5.2 ±0.1	5.3 ±0.2
Phosphatidyl inositol	3.9 ±0.1	3.3 ±0.1	4.0 ±0.1	3.7 ±0.1	3.6 ±0.1	4.3 ±0.3	5.3 ±0.1	6.1 ±0.1	4.1 ±0.2	4.3 ±0.1	4.1 ±0.3
Phosphatidic acid	0.2 ±0.1	0.3 ±0.1	Trace	0.4 ±0.1	0.2	0.1 ±0.05	Trace	0.2 ±0.1	Trace	0.1 ±0.0	0.3 ±0.2
Diphosphatidyl glycerol	10.5 ±0.2	10.6 ±0.0	11.5 ±0.1	11.0 ±0.0	12.6 ±0.0	11.2 ±0.1	12.6 ±0.3	9.0 ±0.1	9.0 ±0.3	8.9 ±0.1	10.1 ±0.1
Phosphatidyl glycerol	1.5 ±0.1	1.0 ±0.1	0.8 ±0.1	0.9 ±0.1	1.0 ±0.1	0.6 ±0.1	0.2 ±0.1	0.6 ±0.1	0.3 ±0.1	Trace	0.2 ±0.1
Lysobisphosphatidic acid	ND[e]	ND	ND	ND	ND	ND	ND	0.2 ±0.1	ND	0.1 ±0.0	0.2 ±0.2
Lysophosphatidyl choline	Trace	0.5 ±0.1	0.3 ±0.1	0.8 ±0.1	0.9 ±0.1	1.6 ±0.1	0.2 ±0.1	3.5 ±0.1	0.5 ±0.2	0.2 ±0.1	0.2 ±0.1
Lysophosphatidyl ethanolamine	ND	ND	ND	ND	ND	ND	ND	1.5 ±0.1	ND	ND	ND
Sphingomyelin	2.7 ±0.1	2.9 ±0.0	2.9 ±0.1	3.3 ±0.1	3.7 ±0.1	3.5 ±0.1	4.7 ±0.3	4.9 ±0.1	7.2 ±0.0	6.4 ±0.2	6.0 ±0.2
Sum	89.3	87.5	85.9	89.9	91.1	93.6	94.3	95.0	91.5	99.0	100.4
P recovery, %	95.7	94.3	93.5	95.7	94.6	96.8	98.0	99.8	95.8	99.9	100.4
Total unidentified	10.7	12.5	14.1	10.1	8.9	6.3	5.6	5.0	8.5	0.9	0.0

[a]Phospholipid values as per cent of total lipid phosphorus ± standard deviation.

[b]As percentage of the fresh weight.

[c]Milligrams of phosphorus per 100 mg of lipid.

[d]Number of TLC determinations of each phospholipid class.

[e]ND, not detected.

TABLE II

Skeletal Muscle Phospholipids[a]

Species No. Animals	Human 1	Bovine 1	Rat 5	Mouse 5	Frog 10	Lobster 8	Scallop 8	Pink Abalone 8	Sea Urchin 8
H_2O, %	72.70	72.57	74.27	74.92	85.21	75.34	76.02	69.56	85.33
Lipid[b], %	10.50	13.01	1.88	7.46	0.42	0.93	0.82	0.93	0.50
Lipid P[c]	0.50	0.31	1.87	1.89	3.23	2.52	2.45	2.28	1.53
No. determinations	8	8	8	8	8	8	8	8	8
Phosphatidyl choline	48.0 ±0.4	46.5 ±0.8	51.1 ±0.5	52.4 ±0.0	55.2 ±0.6	54.6 ±0.6	35.4 ±0.4	40.4 ±0.1	47.5 ±0.2
Phosphatidyl ethanolamine	26.4 ±0.5	26.6 ±0.4	22.2 ±0.0	25.8 ±0.2	29.0 ±0.6	23.6 ±0.4	25.6[d] ±0.0	26.9 ±0.1	23.1 ±0.3
Phosphatidyl serine	3.3 ±0.2	4.1 ±0.2	3.7 ±0.1	4.0 ±0.0	3.4 ±0.1	6.2 ±0.5	11.9 ±0.2	9.9 ±0.3	9.0 ±0.4
Phosphatidyl inositol	8.8 ±0.1	5.6 ±0.3	8.9 ±0.2	6.7 ±0.0	5.5 ±0.2	4.9 ±0.4	5.0 ±0.1	4.4 ±0.1	4.3 ±0.2
Phosphatidic acid	1.2 ±0.3	0.3 ±0.04	0.8 ±0.05	0.1 ±0.05	0.1 ±0.0	0.2 ±0.05	Trace	0.3 ±0.1	0.3 ±0.1
Diphosphatidyl glycerol	6.6 ±0.3	8.9 ±0.2	1.4 ±0.05	5.8 ±0.2	2.3 ±0.3	1.3 ±0.05	1.4 ±0.1	0.6 ±0.1	4.7 ±0.0
Phosphatidyl glycerol	1.0 ±0.2	0.3 ±0.05	0.9 ±0.1	0.7 ±0.2	0.2 ±0.1	Trace	0.1 ±0.05	Trace	0.4 ±0.1
Lysobisphosphatidic acid	Trace	0.1 ±0.05	0.3 ±0.05	Trace	0.1 ±0.0	Trace	0.1 ±0.05	ND	0.1 ±0.05
Lysophosphatidyl choline	Trace	0.7 ±0.1	2.7 ±0.1	0.6 ±0.1	0.4 ±0.1	1.1 ±0.1	0.5 ±0.1	0.7 ±0.1	1.4 ±0.2
Lysophosphatidyl ethanolamine	ND	ND	1.3 ±0.1	Trace	Trace	ND	0.2 ±0.05	ND	ND
Sphingomyelin	4.0 ±0.2	4.5 ±0.2	2.7 ±0.1	3.6 ±0.0	3.1 ±0.3	8.4 ±0.2	ND	ND	ND
Ceramide aminoethyl-phosphonate	ND	ND	ND	ND	ND	ND	16.8 ±0.3	9.3 ±0.2	ND
Ceramide phosphoryl-ethanolamine	ND	ND	ND	ND	ND	ND	ND	ND	3.6 ±0.2
Sum	99.3	97.6	96.0	99.7	99.3	100.3	97.0	92.5	94.4
Other	0.9	1.4	4.4	0.6	0.3	0.0	2.1	6.4[e]	6.4[e]
Recovery, %	100.2	99.0	100.4	100.3	99.6	100.3	99.1	98.9	100.6

[a,b,c]As for Table I.

[d]Phosphatidyl 2-aminoethylphosphonate represented 0.3% ± 0.1% of the lipid phosphorus.

[e]About one half of the other phosphorus (unidentified components) remained at or near the point of application.

Invertebrate skeletal muscle phospholipid class composition (Table II) differs qualitatively and/or quantitatively from vertebrate muscles. Although sphingomyelin is present in lobster muscle, this phospholipid was not present in other invertebrate species examined. Ceramide aminoethylphosphonate was found to replace sphingomyelin in two species (scallop, abalone) and in another (sea urchin), sphingomyelin was replaced by ceramide phosphorylethanolamine, the phosphodiester analogue of ceramide aminoethylphosphonate. Rather consistent quantitative differences between vertebrate and invertebrate skeletal muscles are the higher phosphatidyl serine and lower phosphatidyl inositol values of invertebrate muscles. Three of the four invertebrate species examined also had a much higher sphingolipid level, although the level of ceramide phosphorylethanolamine in the sea urchin was lower and similar to that of sphingomyelin of vertebrate skeletal muscle.

DISCUSSION

The validity of phospholipid class values should be judged in the light of possible effects of post-mortem enzymatic alteration and the accuracy of methods for determination of lipid classes (1). There is little enzymatic degradation of vertebrate heart muscle phospholipid even after standing at room temperature for several hours (9). Similar results were obtained with vertebrate skeletal muscle (Simon, in preparation). Changes of this type were prevented in our studies of invertebrates by freezing within 3 min of the time animals were removed from the ocean. Alteration of lipids prior to analysis was prevented by addition of the antioxidant butylated hydroxytoluene and use of the solvent replacement procedure as previously described (1) rather than evaporation to complete dryness. Precision (reproducibility) of the procedures was documented by small values for standard deviations. Lipid classes were identified by TEAE column chromatography of the intact lipids, TLC migration, the colors obtained with ninhydrin and the formaldehyde-sulfuric acid reagent with which sterols and glycolipids give specific colors before charring (pink and purple, respectively), and identification of hydrolysis products.

Phospholipids appear to be present almost exclusively in organs as major components of the cell surface and subcellular particulate membranes (10,11). Each membrane when isolated in highly purified and undegraded form appears to have a characteristic and rather reproducible phospholipid class distribution which differs qualitatively and/or quantitatively from that of other membranes (4,10,11). The phospholipid composition of an organ is thus determined in part by the relative abundance of each type of membrane. Data with maximum significance for species variations of membrane phospholipid class distribution are thus obtainable only from organs composed almost entirely of one type of cell with the same function in all species to be compared. The organ must also have a relatively constant proportion of the different subcellular particulates. Muscle fulfills these criteria very well and the relatively more abundant skeletal muscle is particularly good for studies of invertebrates of small size. The data obtained for vertebrate muscle indicate that there is little variation in the proportions of the different types of membranes of muscle cells and that there is little or no species variability in the phospholipid class distribution of vertebrate muscle cell surface and subcellular particulate membranes. This conclusion is supported by direct analysis of highly purified mitochondria from bovine, human and rat hearts (4) which were found to have virtually identical phospholipid class distributions. It is also in agreement with results obtained for other vertebrate organs (1-5).

Direct, accurate determination of the true in vivo phospholipid composition of subcellular particulates is difficult. Procedures for isolation and characterization of pure subcellular particulates are relatively time-consuming and must be carefully chosen and modified specifically for a particular organ or species (10,11). Furthermore, enzymes may act on lipids during subcellular particulate isolation. Whole organ analysis thus provides a valuable baseline for subcellular particulate work.

The data for invertebrates show greater qualitative and quantitative variations and it was not possible to determine to what extent the variations arose from differences in the types of cells present, the relative proportions of different membranes, and variability of composition of the individual membranes.

In previous studies, Hack et al. (12) investigated the qualitative lipid class patterns of invertebrates by one-dimensional paper chromatography, but failed to observe many of the lipid classes we have encountered. They did, however, demonstrate the presence of plasmalogen (vinyl ether linked) forms of glycerolphospholipids in various species in agreement with the earlier report of Rapport and Alonzo (13). In molluscan tissue, ether linked hydrocarbon chains were shown (14) to be very abundant in glycerolphospholipids. In an informative series of publications (15-17), De Koning noted the presence of ceramide amino-

ethylphosphonate [first isolated and characterized from the sea anemone (6,8)] in the abalone, *Haliotis midae*. Ceramide aminoethylphosphonate was not found in two species of fish and the rock lobster (*Jasus lalandii*). The presence of ceramide phosphorylethanolamine in tissues of some invertebrates has been firmly established by Hori et al. (18). The presence of a very small amount of the phosphonic acid analogue of phosphatidyl ethanolamine in *Tetrahymena pyriformis* was established by Liang and Rosenberg (19).

Of special interest with regard to differences in membrane phospholipid class composition of organelles from vertebrates and invertebrates are the phospholipid data presented by Thomas and Gilbert (20) who reported for moth (*Hyalophora cecropia*) sarcosomes (mitochondria) data closely similar to that reported for beef heart mitochondria (10,11). This close agreement indicated that, in general, subcellular particulates from vertebrates and invertebrates may have the same lipid class compositions. The phospholipid values for insect mitochondria obtained in other investigations (21,22) do not agree with those reported by Thomas and Gilbert (20), but the differences appear to us to be attributable to the superior methodology used by Thomas and Gilbert.

ACKNOWLEDGMENTS

Supported by U.S. Public Health Service Grants NB06237, NB06113 and NB07760.

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[Received January 22, 1969]

Hepatic Metabolism of 1-^{14}C Octanoic and 1-^{14}C Margaric Acids

ABSTRACT

The hepatic metabolism of 1-^{14}C margaric acid, a 17 carbon long chain saturated fatty acid which is present in the liver in trace amounts, was compared with 1-^{14}C octanoic acid and 1-^{14}C palmitic acid to determine if the enhanced oxidation of medium chain fatty acids to CO_2 was dependent on fatty acid chain length or the endogenous pool size of the fatty acid substrate. Despite the fact that endogenous margarate is present in trace amounts, there was no significant difference in the oxidation of margarate and palmitate to CO_2, while the oxidation of octanoate to CO_2 was significantly more rapid. Both margarate and palmitate were more readily incorporated into lipid soluble products in contrast to the low rate of incorporation of octanoate. However, margarate was less readily incorporated into triglyceride, phospholipid and monoglyceride than palmitate. These studies suggest that the chain length rather than hepatic content of the fatty acid determines whether the carboxyl group of equimolar amounts of a 1-^{14}C-carboxyl labeled fatty acid will be preferentially oxidized to CO_2 or incorporated into tissue lipid in the liver.

Previous studies in rat liver slices showed that octanoic acid, a constituent of medium chain triglycerides, is more readily oxidized to CO_2 and less effectively incorporated into tissue lipids than an equimolar amount of palmitic acid, a constituent of long chain triglycerides (1). These differences in metabolism have also been demonstrated in other tissues, namely, kidney, skeletal muscle, myocardium, brain, intestine and adipose tissue (2,3). Moreover, studies in both the intact and hepatectomized rat have corroborated the in vitro differences in the rate of oxidation of octanoate and palmitate. It is known, however, that palmitic acid is one of the most prevalent fatty acids in tissue lipids. For example, 23% of the fatty acids in liver is palmitate, while octanoate is present in only trace amounts (< 0.5%) (4). Therefore, the possibility exists that the results of the above studies are due to unequal dilution of the substrate label by endogenous fatty acid in tissue lipid.

To test this hypothesis, we have compared the hepatic metabolism of 1-^{14}C octanoate and 1-^{14}C palmitate with 1-^{14}C margarate, a 17

TABLE I

μMoles Fatty Acid 1-^{14}C Incorporated into Lipid/g Liver[a]

Time	Octanoate	Palmitate	Margarate
1 hr	24 ± 3	171 ± 35	181 ± 11
2 hr	56 ± 29	306 ± 50	307 ± 19

[a]The mean and standard deviation are representative of three separate experiments.

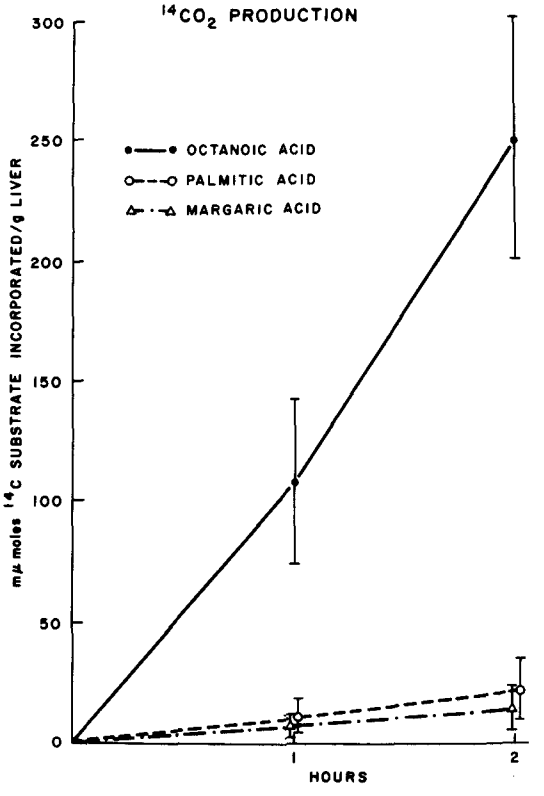

FIG. 1. Oxidation of 1-^{14}C fatty acid substrate to $^{14}CO_2$ by rat liver slices at 1 and 2 hr. The mean and standard deviation represent three separate experiments.

TABLE II

Lipid-Soluble Products From 1-^{14}C Margaric and 1-^{14}C Palmitic Acid by Liver Slices[a]

^{14}C Substrate (3μmoles)	Percentage distribution of total lipid radioactivity		
	Phospholipid and monoglyceride	Unesterified fatty acid	Triglyceride
Margaric Acid (6)	29.0±3.8	42.8±9.8	25.0±9.8
Palmitic Acid (6)	34.5±2.4	16.7±2.0	46.0±3.4
p value	<0.05	<0.001	<001

[a]Lipid soluble products migrating with diglycerides comprised 1-2% of activity for palmitate and 2-3% for margarate. Cholesterol and cholesterol esters accounted for less than 2% of the activity for both substrates. Numbers in parenthesis refer to the number of experiments. Incubations were for 2 hr and results are expressed as the mean ± standard deviation.

carbon, straight chain saturated fatty acid which is present in the liver, but in concentrations similar to octanoate.

Incubations of liver slices (.344±0.02 g) from male, Sprague-Dawley rats in pH 7.4 Krebs Ringer bicarbonate buffer (1/2 calcium) containing 5% albumin and 3 μmoles of the ^{14}C fatty acid substrate were performed as previously described (1). 1-^{14}C Octanoic and 1-^{14}C palmitic acids were purchased from New England Nuclear Corp., Boston, Mass. and 1-^{14}C margaric acid from Nuclear-Chicago, Des Plaines, Ill. After 1 and 2 hr, the radioactivity contained in both CO_2 and in the tissue lipid was determined by liquid scintillation spectroscopy. The CO_2 was trapped in hyamine hydroxide and tissue lipid was extracted with chloroform-methanol. In addition, in order to ascertain if margarate was incorporated as readily into the same lipid classes as palmitate, the per cent distribution of radioactivity into lipid soluble products was determined for both margarate and palmitate by thin layer chromatography as previously described (1).

The results (Fig. 1) indicate that the rate of oxidation of margarate is similar to that of palmitate, in spite of marked differences in their tissue concentration. However, the rate of conversion of the carboxyl label of octanoate to $^{14}CO_2$ is over 10 times greater than that of either palmitate or margarate, differences that were statistically significant ($p < 0.01$). In contrast, both margarate and palmitate are readily incorporated into tissue lipid compared to octanoate (Table I). Although margarate and palmitate were both incorporated into lipid soluble products of rat liver slices to a similar extent, the distribution of the fatty acids into the different lipid classes was significantly different (Table II). Margarate was less readily incorporated into triglyceride, phospholipid and monoglyceride. Furthermore, nearly half of margarate (42.8 ± 9.8%) but only 16.7 ± 2.0% of palmitate remained in the unesterified fatty acid fraction after the 2 hr incubation. In this respect, margarate behaved more like octanoate (1) although the total incorporation of margarate into tissue lipid was considerably greater.

Since margarate is a long chain saturated fatty acid, like palmitate, it might also be diluted by endogenous saturated fatty acids of similar although not identical chain lengths, despite its own very low endogenous concentration in hepatic lipids. This seems quite unlikely however, since there were distinct differences in the distribution of the two long chain fatty acids in the lipid soluble product (Table II). Also, when the amounts of margarate or palmitate in the incubation were reduced by a factor of 3-10, or the weight of the liver slices was altered by a factor of 0.5 to 2.0, the amount of 1-$^{14}CO_2$ recovered was directly proportional (Boyer and Scheig, unpublished observations). These results would not be expected if there was significant dilution of either labeled fatty acid substrate by endogenous fatty acids pools.

These results suggest, therefore, that the chain length of the saturated fatty acid substrate determines whether the carboxyl group of the fatty acid will be principally oxidized or incorporated into tissue lipid, and excludes the possibility that the observed low rate of oxidation of long-chain saturated fatty acids is due to dilution of the isotope by unlabeled tissue lipid. Since the concentration of unbound fatty acid in the medium may be an important determinant of the rate of its metabolism (5), the possibility exists that our results might be explained by differences in the binding affinity of the fatty acid substrate to albumin or in its water solubility. However, our data indicate that the total amount of substrate incorporated

into hepatic lipid soluble product and metabolized to CO_2 is similiar for all three fatty acids. After 2 hr, for example, 311 μmoles of octanoate/g liver was either oxidized or incorporated into tissue lipid compared with 330 μmoles of palmitate/g liver and 323 μmoles of margarate/g liver. Thus it seems that any differences in the amount of unbound fatty acid in the medium could not account for our observations.

JAMES L. BOYER
ROBERT SCHEIG
Department of Internal Medicine
Yale University School of Medicine
New Haven, Connecticut 06510

ACKNOWLEDGMENTS

Supported by Grants AM 05966 and AM 5180 from the National Institutes of Health; technical assistance, Rose Marie Moquin.

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[Received May 15, 1969]

A Cyanogenetic Lipid from *Cordia verbenacea* DC. Seed Oil

ABSTRACT

Cyanogenetic nonglycerol diesters, which are composed of two fatty acid moieties esterified with an unsaturated five-carbon dihydroxynitrile, constitute 35% of *Cordia verbenacea* DC. (Boraginaceae) seed oil.

The only cyanogenetic lipid reported extensively in the literature occurs in seed oil of *Schleichera trijuga* (Sapindaceae). It has variously been referred to as nonglucosidic (1), glucosidic (2), a glycerol derivative (3) and a nonglycerol ester (4), but its structure or that of any other cyanogenetic lipid has not been reported. We wish to describe our investigation of the structure of a cyanogenetic lipid isolated in 35% yield from seed oil of *Cordia verbenacea* DC. (Boraginaceae).

The oil was fractionated by countercurrent distribution between hexane and nitroethane in a 200 tube apparatus. In this solvent system, the nitrogen containing lipid fraction (NCLF) traveled as one peak, which moved slower than triglyceride constituents of the oil.

When analyzed by temperature-programmed gas liquid chromatography (GLC) on a 0.3 X 19.7 cm stainless steel column packed with 3% OV-1 on Gaschrom Q (Applied Science Laboratories), the NCLF separated into four peaks with elution temperatures intermediate between those of ethylene glycol distearate and triglycerides of long chain acids. Adjacent peaks appeared to differ by two carbon units. On thin layer chromatography (TLC) with Silica Gel G, developed in hexane-ether (95:5), the NCLF migrated as one spot slightly ahead of ordinary triglycerides. The infrared (IR) spectrum (1% in CS_2) of the NCLF had broad, medium intensity bands at 938 and 1015 cm^{-1} possibly due to terminal methylene and allylic ester groupings. The ester carbonyl band at 1760 cm^{-1} was slightly broadened; otherwise, the IR spectrum resembled those of ordinary triglycerides. The ultraviolet spectrum of the NCLF in cyclohexane had no absorption maxima above 210 mμ. Optical rotatory dispersion measurements showed that the NCLF was dextrorotatory, $[\alpha]_{500}^{26^{\circ}C} = +1.4^{\circ}$ ($c = 2.1$, hexane).

Elemental analysis revealed that this unusual ester contained 2.2% of nitrogen (Dumas). Treatment with dilute base at 35-50 C for 30 min produced HCN as shown by positive picrate (5) and Prussian blue (6) tests. These color reactions were greatly accelerated by acidification of the basic test solution. The lack of nitrile absorption in the IR spectrum is not

[1]Presented at the AOCS Meeting, April 1969, San Francisco, Calif.

[2]Postdoctoral Research Associate, 1967-68. Present address: Department of Botany, University of Texas, Austin.

[3]Department of Chemistry, University of Arizona, Tucson.

into hepatic lipid soluble product and metabolized to CO_2 is similiar for all three fatty acids. After 2 hr, for example, 311 μmoles of octanoate/g liver was either oxidized or incorporated into tissue lipid compared with 330 μmoles of palmitate/g liver and 323 μmoles of margarate/g liver. Thus it seems that any differences in the amount of unbound fatty acid in the medium could not account for our observations.

JAMES L. BOYER
ROBERT SCHEIG
Department of Internal Medicine
Yale University School of Medicine
New Haven, Connecticut 06510

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Cyanogenetic nonglycerol diesters, which are composed of two fatty acid moieties esterified with an unsaturated five-carbon dihydroxynitrile, constitute 35% of *Cordia verbenacea* DC. (Boraginaceae) seed oil.

The only cyanogenetic lipid reported extensively in the literature occurs in seed oil of *Schleichera trijuga* (Sapindaceae). It has variously been referred to as nonglucosidic (1), glucosidic (2), a glycerol derivative (3) and a nonglycerol ester (4), but its structure or that of any other cyanogenetic lipid has not been reported. We wish to describe our investigation of the structure of a cyanogenetic lipid isolated in 35% yield from seed oil of *Cordia verbenacea* DC. (Boraginaceae).

The oil was fractionated by countercurrent distribution between hexane and nitroethane in a 200 tube apparatus. In this solvent system, the nitrogen containing lipid fraction (NCLF) traveled as one peak, which moved slower than triglyceride constituents of the oil.

When analyzed by temperature-programmed gas liquid chromatography (GLC) on a 0.3 X 19.7 cm stainless steel column packed with 3% OV-1 on Gaschrom Q (Applied Science Laboratories), the NCLF separated into four peaks with elution temperatures intermediate between those of ethylene glycol distearate and triglycerides of long chain acids. Adjacent peaks appeared to differ by two carbon units. On thin layer chromatography (TLC) with Silica Gel G, developed in hexane-ether (95:5), the NCLF migrated as one spot slightly ahead of ordinary triglycerides. The infrared (IR) spectrum (1% in CS_2) of the NCLF had broad, medium intensity bands at 938 and 1015 cm^{-1} possibly due to terminal methylene and allylic ester groupings. The ester carbonyl band at 1760 cm^{-1} was slightly broadened; otherwise, the IR spectrum resembled those of ordinary triglycerides. The ultraviolet spectrum of the NCLF in cyclohexane had no absorption maxima above 210 mμ. Optical rotatory dispersion measurements showed that the NCLF was dextrorotatory, $[\alpha]_{500}^{26^{\circ}C} = +1.4^{\circ}$ ($c = 2.1$, hexane).

Elemental analysis revealed that this unusual ester contained 2.2% of nitrogen (Dumas). Treatment with dilute base at 35-50 C for 30 min produced HCN as shown by positive picrate (5) and Prussian blue (6) tests. These color reactions were greatly accelerated by acidification of the basic test solution. The lack of nitrile absorption in the IR spectrum is not

[1]Presented at the AOCS Meeting, April 1969, San Francisco, Calif.

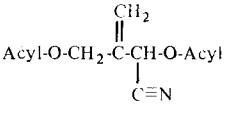
[2]Postdoctoral Research Associate, 1967-68. Present address: Department of Botany, University of Texas, Austin.

[3]Department of Chemistry, University of Arizona, Tucson.

unusual for compounds having an oxygen function (especially an ester) close to the nitrile grouping (7).

Saponification of the NCLF (methyl laurate included as internal standard) yielded a mixture of ordinary fatty acids of C_{16}, C_{18}, C_{20} and C_{22} chain lengths, 50% of which was C_{20} monoene. Neither glycerol nor ethylene glycol was present in the hydrolyzate as shown by TLC. By GLC analysis of the esterified fatty acid mixture, we demonstrated that two fatty acid moieties are incorporated per molecule of the NCLF. The extremely unstable alcohol moiety has not been isolated intact.

The 100 MHz nuclear magnetic resonance (NMR) spectrum of the NCLF in $CDCl_3$ (reference, tetramethylsilane) is in accord with structure I, where acyl groups are long chain fatty acids.



[I]

Signals expected from protons of ordinary fatty acids are present. These include terminal methyl protons (τ 9.15, triplet), shielded methylene protons (τ 8.75, singlet), protons on carbons α to double bonds (τ 8.05, multiplet), and olefinic protons (τ 4.72, rough triplet). In $CDCl_3$ the signal due to protons on carbons α to carboxyl groups approximates a quartet centered at τ 7.66 (4H), but in benzene-d_6 this signal becomes a pair of overlapping triplets centered at τ 7.64 and 7.68 (J = 7 Hz). This nonequivalence of the methylene groups α to fatty acid carboxyl groups in structure I is probably caused by influence of the nitrile grouping on one of them.

Additional signals (all apparent singlets) appear in the spectrum ($CDCl_3$) at τ 5.37 (2H), τ 4.50 (1H), τ 4.34 (1H) and τ 4.06 (1H). On examination in benzene-d_6, however, the signal at τ 5.37 becomes two overlapping doublets centered at τ 5.44 and 5.60 (J = 13 Hz). These doublets are due to the two nonequivalent protons of the methylene group adjacent to oxygen in the dihydroxynitrile moiety. The signal at τ 4.06 ($CDCl_3$) is assigned to the proton attached to the cyanohydrin carbon. Terminal methylene protons frequently are nonequivalent and show small coupling constants (8,9), therefore, the apparent singlets at τ 4.34 and 4.50 arise from these protons in structure I. Decoupling experiments show a number of long range couplings that are not readily apparent in the original spectrum. Irradiation at τ 5.37 sharpens all three downfield signals, but fine splitting in these signals indicates that long range allylic coupling (10) also occurs between the terminal methylene protons and the proton on the cyanohydrin carbon.

By random distribution, the most likely detectable combinations of two acyl groups, which would give the largest molecular weights for components of the NCLF, are a C_{20} monoene plus either a C_{22} saturate or monoene. If we assume a diol of molecular weight 113 (from NMR data), the largest components of the NCLF should have a molecular weight of 725 or 727. The mass spectrum of the NCLF revealed that the largest fragments had m/e 725 and 727, and also showed other molecular ions at m/e 671, 673, 697 and 699 due to other combinations of two acyl groups.

Preliminary data indicate that GLC and IR characteristics of *Cardiospermum halicacabum* (Sapindaceae) seed oil are nearly identical with those of *Cordia* oil. Therefore it is possible that the material isolated by Hopkins et al. (11) from this seed oil (after chemical treatment) may be related to the dihydroxynitrile moiety of I. Similarly, the IR of *Schleichera trijuga* seed oil (4) resembles that of *Cordia* with the exception that nitrile absorption is observed. Indian workers (4) have recently isolated β-methyl α-tetronic acid from chemically treated, unfractionated *Schleichera* oil, and we have also isolated this compound, along with other artifacts, from products derived by chemical treatment of the *Cordia* NCLF.

Additional results from our continuing investigation of these nitrogen containing lipids will be reported later.

K. L. MIKOLAJCZAK
D. S. SEIGLER[2]
C. R. SMITH, JR.
I. A. WOLFF
R. B. BATES[3]
Northern Regional Research Laboratory
Agricultural Research Service
U.S. Department of Agriculture
Peoria, Illinois 61604

ACKNOWLEDGMENTS

NMR spectra by D. Weisleder and L. W. Tjarks, GLC analyses by J. W. Hagemann, mass spectra by W. K. Rohwedder, elemental analyses by Mrs. C. E. McGrew and Mrs. B. R. Heaton, and seed samples supplied by A. S. Barclay, USDA, Crops Research Division, Beltsville, Md.

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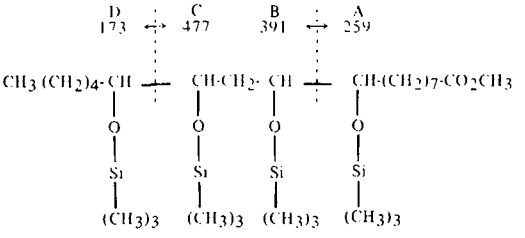
[Received June 24, 1969]

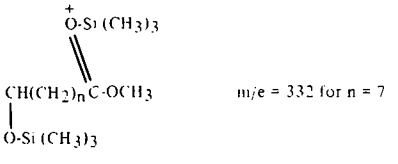
Determination of Double Bond Position in Polyunsaturated Fatty Acids Using Combination Gas Chromatography Mass Spectrometry

The use of gas liquid chromatography combined with mass spectrometry (GLC-MS) for the determination of the double bond position in unsaturated fatty acids has been limited since unsaturated compounds generally give similar spectra. A method for the determination of the double bond position in monoenoic fatty acids employing the trimethyl silyl ether derivatives of the corresponding hydroxy esters has recently been presented by us (1) as well as others (2,3). In this communication, an extension of that method to the determination of double bond position of dienoic and trienoic fatty acid methyl esters using combined GLC-MS is presented. Polyhydroxy fatty acid methyl esters were synthesized as previously described (1), except that the extraction of the aqueous layer with chloroform was repeated three more times to ensure complete extraction of the polyhydroxy derivatives. The preparation of the silyl ether derivatives and their subsequent GLC separation and MS analysis were carried out as previously described (1). Samples of methyl linoleate, methyl α-linolenate, and methyl γ-linolenate were obtained commercially (The Hormel Institute). A sample of methyl 9,15-octadecadienoate was furnished by H. J. Dutton, NRRL, USDA.

A summary of the principal fragments obtained from the MS of the derivatives obtained from methyl linoleate, α-linolenate, γ-linolenate and 9,15-octadecadienoate is given in Table I. These indicate the position of the original double bond. In all cases fragmentation occurred between adjacent substituent groups giving rise to ions designated as A, B, C and D in the case of the linoleate derivative which indicate the position of the double bond directly:



In all cases, several other ions were observed which also may be used to indicate the double bond position. A rearrangement ion was present in all of the spectra (at m/e 332 in the case of derivatives formed from double bond at the 9 position). This ion has the following proposed structure (2) which is also in agreement with our data:



The mass spectrum of the corresponding trimethyl silyl ether of ethyl 9,10-dihydroxy octadecanoate yielded an ion of 14 units higher mass, thus confirming the presence of the ester group in this ion. High resolution mass measure-

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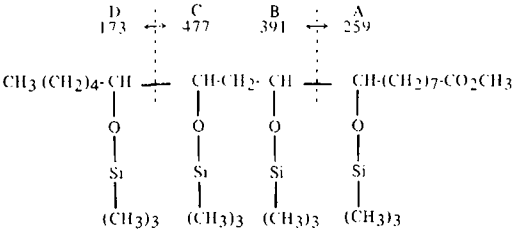

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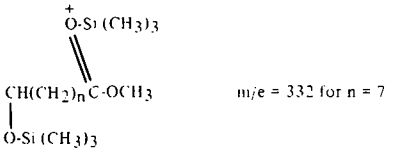

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TABLE 1

Mass Spectral Fragmentation of the Trimethylsilyl Ether Derivative of Hydroxylated Polyunsaturated Fatty Acid

m/e	Fragment	Per cent of base peak	Double bond position	m/e	Fragment	Per cent of base peak	Double bond position
Linoleic acid ($\Delta^{9,12}$)				Linoleic acid ($\Delta^{9,15}$)			
259	A	37.0	9	259	A	100.0	9
169	A-90	0.5		169	A-90	2.0	
271	A+102-90	6.0		271	A+102-90	2.0	
332	A+73	4.0		332	A+73	27.0	
361	A+102	5.0		361	A+102	1.0	
391	B	4.0	9	391	B	2.0	9
301	B-90	97.0		301	B-90	23.0	
211	B-2X90	7.1		211	B-2X90	25.2	
121	B-3X90	0.5		121	B-3X90	7.0	
477	C	1.0	12	493	B+102	1.5	
387	C-90	100.0		519	C	9.0	15
297	C-2X90	9.0		429	C-90	13.0	
207	C-3X90	1.0		339	C-2X90	33.0	
173	D	17.5	12	249	C-3X90	9.5	
275	D+102	10.0		131	D	25.0	15
185	D+102-90	1.0					
α - Linolenic acid ($\Delta^{9,12,15}$)				γ - Linolenic acid ($\Delta^{6,9,12}$)			
259	A[a]	100.0	9	217	A	41.5	6
169	A-90[b]	31.2		127	A-90	4.3	
271	A+102-90	81.5		229	A+102-90	18.1	
332	A+73	6.0		290	A+73	3.3	
361	A+102	22.0		319	A+102	4.3	
567	B	---	9	609	B	---	6
477	B-90	---		517	B-90	---	
387	B-2X90	3.5		429	B-2X90	26.5	
297	B-3X90	3.0		339	B-3X90	19.1	
207	B-4X90	4.0		249	B-4X90	2.4	
117	B-5X90	7.0		333	C	1.1	9
477	C	---	12	243	C-90	10.9	
387	C-90	3.5		435	C+102	3.5	
297	C-2X90	3.0		493	D	0.3	9
207	C-3X90	4.0		403	D-90	1.7	
349	D	3.0	12	313	D-2X90	7.9	
259	D-90	---		223	D-3X90	0.9	
169	D-2X90	---		653	E	---	12
131	F	38.5	15	563	E-90	---	
233	F+102	44.0		473	E-2X90	20.9	
143	F+102-90	25.0		383	E-3X90	19.8	
				293	E-4X90	7.7	
				173	F	22.6	12
				83	F-90	1.1	
				275	F+102	13.6	
				185	F+102-90	4.2	

[a]Contribution from the D-90 fragment.

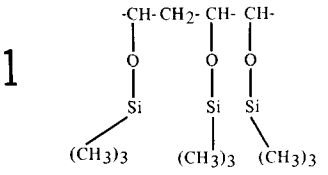
[b]Contribution from the D-2X90 fragment.

ments indicated that the postulated ion has the suggested molecular formula (calculated mass 332.2193, found 332.2189).

In addition to the rearrangement ion several other series of ions are present which correspond to the loss of trimethylsilanol from a fragment formed through the rupture of the bond between the substituent bearing carbon atoms of the derivative (i,e., M-B+90, or the fragment A-90). This consecutive loss of trimethylsilanol from various fragments continues to produce several series of ions which may in general be represented as: $(M-(X-90))^+$, $(M-(X-2X90))^+$, $(M-(X-3X90))^+$ and $(M-(X-4X90))^+$, where X may be A, B, C, etc. Another series of ions formed by cleavage α to the bond that is substituted was present in cases where there was a methylene group situated between the substituted carbon atoms (i.e., $B+102^+$).

In addition to the ions discussed above, an ion of m/e = 191 was present in all of the deri-

vatives containing the grouping (1): However, its intensity was high only in those derivatives which had more than three carbon atoms between any two carbons bearing the trimethylsilyloxy groups. (Linoleic acid $\Delta^{9,12}$, 2.0%; linoleic acid $\Delta^{9,15}$, 64.0%; α-linolenic acid $\Delta^{9,12,15}$, 28.0%; γ-linolenic acid $\Delta^{6,9,15}$, 74.8%):



A cyclic ion whose structure cannot be assigned at this time could be the precursor of the ion at m/e 191: $[(CH_3)_3Si\text{-}O\text{-}\overset{+}{C}H\text{-}O\text{-}Si\text{-}(CH_3)_3]$. A similar ion has been postulated by Niehaus and Ryhage (4) for the m/e 75 ion derived from the corresponding polymethoxy ether derivatives.

E. G. PERKINS
C. J. ARGOUDELIS
Department of Food Science
The Burnsides Research Laboratory
The University of Illinois
Urbana, Illinois 61801

ACKNOWLEDGMENT

This work was supported by research grant UI 00115 from the National Center for Urban and Industrial Health and grant AM 00257 from the U.S. Public Health Service. D. Jerina, NIAMD, NIH, provided high resolution mass measurements.

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[Received March 20, 1969]

Reciprocal Interactions in the Desaturation of Linoleic Acid into γ-Linolenic and Eicosa-8,11,14-Trienoic into Arachidonic

ABSTRACT

Variable concentrations of [$I^{14}C$] linoleic acid and [$I^{14}C$] eicosa-8,11,14-trienoic acid were incubated with liver microsomes in a medium containing the necessary cofactors for fatty acid desaturation. The conversion of linoleic into γ -linolenic acid and eicosatrienoic into arachidonic acid were mutually inhibited and the inhibition depended on the concentration of the fatty acids incubated.

In studying the regulation (1) of unsaturated fatty acid biosynthesis in the microsomes of the liver, linoleic acid desaturation into γ -linolenic acid was inhibited by other members of the linoleic acid family, such as γ -linolenic and arachidonic acid (2). This effect was evoked either when the acids were tested as CoA derivates or as free acids plus ATP, CoA and Mg^{++}. In order to understand more properly the interrelationships of the members of this series, the effect of [$I^{14}C$]-linoleic acid desaturation to γ -linolenic acid upon [$I^{14}C$]-eicosa-8,11,14-trienoic acid conversion to arachidonic acid and vice versa was studied.

Liver microsomes of male albino rats fed a balanced diet were separated by differential centrifugation at 140,000 X *g* (3). Microsomal protein 5 mg and 200 to 800 nmoles [$I^{14}C$]-linoleic acid (specific activity 52.9 mC/mmole, diluted with unlabeled acid in the proportion 1:144) (The Radiochemical Centre, Amersham, England) were incubated during 20 min at 25 C in air with 200 to 800 nmoles, [$I^{14}C$]-eicosa-8,11,14-trienoic acid (specific activity 1.65 X 10^5 dpm/μmole, synthesized in W. Stoffel's laboratory, Institut fur Physiologische Chemie, Cologne, Germany). The free acids dissolved in propylene-glycol were added to a medium containing in μmoles: $ATPNa_2$, 8; CoA, 1.2; NADPH 2.5; $MgCl_2$ 15; glutathion 4.5; NaF 125; nicotinamide 1; phosphate buffer

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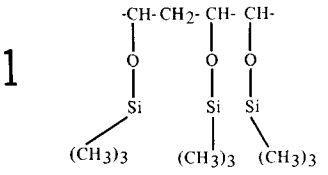

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TABLE I

Effect of Increasing Concentration of Substrate Upon the Conversion of [$I^{14}C$]-Linoleic Into γ-Linolenic Acid and [$I^{14}C$]-Eicosa-8,11,14-Trienoic into Arachidonic Acid[a]

Original substance	Labeled acid Incubated, nmoles	Conversion %	Amount converted, nmoles
[$I^{14}C$]-	200	4.7	9.4
Linoleic	400	2.1	8.4
acid	800	0.7	5.6
[$I^{14}C$] Eicosa-	200	14.8	29.6
8,11,14-trienoic	400	7.2	28.8
acid	800	3.2	25.6

[a]Two hundred nmoles eicosa-8,11,14-trienoic or linoleic acid were included in the medium. Results are the mean of two experiments.

(pH 7.2) 125 in a total volume of 3 ml of 0.15 M KCl; 0.25 M sucrose solution. The amount of fatty acids relative to amount of microsomal protein was so chosen to assure that the fatty acid desaturation was the limiting factor of the reaction and to avoid alien activating effects (1). Once the incubation was stopped the fatty acids were saponified, extracted and esterified with 3 N HCl in methanol, in the usual way (3). The fatty acids were separated by gas liquid chromatography in a Pye apparatus equipped with a column packed with 10% diethylene-glycol succinate in Chromosorb W (100-120 mesh). Appropriate fractions were collected every minute in vials containing the scintillation counting solution and the radioactivity measured in a Packard Tricarb Scintillation Counter.

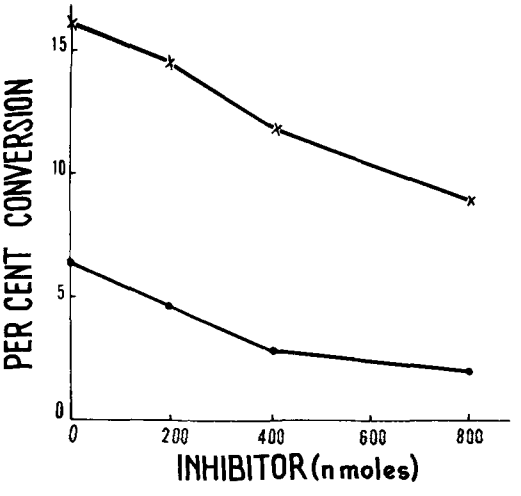

FIG. 1. Mutual effects of the conversion of [$I^{14}C$]-linoleic acid into γ-linolenic acid and [$I^{14}C$]-eicosa-8,11,14-trienoic acid into arachidonic acid. (•—•) 200 nmoles [$I^{14}C$]-linoleic acid were incubated with increasing amounts of eicosa-8,11,14-trienoic acid. (X—X) 200 nmoles [$I^{14}C$]-eicosa-8,11,14-trienoic acid were incubated with increasing amounts of linoleic acid. The experimental conditions are described in the text. Figures are the mean of two experiments.

Figure 1 shows that eicosa-8,11,14-trienoic acid conversion into arachidonic acid (16.1%) is higher than linoleic acid desaturation into γ-linolenic acid (6.4%). These conversions agree with the results found by Stoffel and Schiefer (4). However, the decrease in the conversion of [$I^{14}C$]-linoleic into γ-linolenic acid by effect of substrate concentration is higher than was expected by simple dilution, as is shown in Table I. This result might suggest the possibility of an inhibition provoked by the substrate. The desaturation of eicosa-8,11,14-trienoic acid into arachidonic acid, is also inhibited by its own substrate, suggesting the same possibility.

Eicosa-8,11,14-trienoic acid provoked, as expected, an inhibition of linoleic acid desaturation into γ-linolenic acid and corroborates our postulate that higher members of linoleic acid series may compete with linoleic acid desaturation. This effect is provoked, in suitable conditions, either when the inhibitor is desaturated as is the case with eicosa-8,11,14-trienoic acid or when it is not desaturated as was found with γ-linolenic or arachidonic acid (1,2). The conversion of eicosa-8,11,14-trienoic acid into arachidonic acid is also inhibited by increasing concentrations of linoleic acid (Fig. 1). Marcel et al. (5) have just reported that the addition of linoleic acid to eicosa-8,11,14-trienoic acid in a 2:1 ratio increased the desaturation of the latter by 300%. This result, which in the first instance appears to contradict our finding, must be carefully analyzed. The experimental conditions chosen by the aforementioned authors, that is the ratio 10 mg microsomal protein to 100 nmoles of fatty

acid, appears to be too high to assure that the desaturating enzyme is the limiting factor of the reaction, as we have shown in a previous work (1). Consequently, according to our theory that desaturation and esterification compete for the fatty acid, activating effects were very probably expected to be found in these conditions by addition of another fatty acid that would decrease the amount of substrate available for the esterification. Therefore this result and the other reported activating effects of the addition of γ -linolenic acid, eicosa-11,14-dienoic acid or arachidonic acid to the eicosa-8,11,14-trienoic acid system supports our theory (1).

The fact that eicosa-8,11,14-trienoic acid desaturation to arachidonic acid competes with linoleic acid conversion into γ -linolenic acid and vice versa, does not necessarily prove that the same enzyme or enzymes are used by both acids.

R. R. BRENNER
Catedra de Bioquimica
Instituto de Fisiologia
Facultad de Ciencias Medicas
La Plata, Argentina

ACKNOWLEDGMENTS

Part of this work was done in the Institut fur Physiologische Chemie, Cologne, Germany through the Deutcher Akademischer Austauschdienst and the Consejo Nacional de Investigaciones Cientificas y Tecnicas, Argentina. W. Stoffel provided the drugs and equipment used in this experiment and Mrs. E. C. T. de Serres provided technical assistance. This work has been supported by Grant 9 of the Comision de Investigacion Cientifica, University of La Plata.

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[Received April 22, 1969]

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ABSTRACT

Extracts of spinal cord lipids from normal pigs and littermates affected with myoclonia congenita were examined for lipid and fatty acid composition at 6, 10, 14, 21 and 30 days of age. A marked decrease in total crude lipid and in phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylinositol (PI) occurred in spinal cord lipids of affected pigs. Cholesterol esters were absent from spinal and lipid extracts and no qualitative difference in ganglioside patterns were observed between normal and affected pigs at any age. Spinal cord lipid extracts from affected pigs had less oleic acid except at 30 days and more arachidonic, docosapentaenoic and docosahexaenoic acids than normal.

Previous studies (1,2) indicated that swine affected with myoclonia congenita exhibited variable degrees of hypomyelinization, especially of the spinal cord, as determined by histochemical staining procedures. By ablation techniques it was established that the myoclonus was activated by neural inputs from the brain or spinal nerves but the mechanism was localized at the spinal cord level. The present investigation was developed to survey the phospholipid class composition and the fatty acids of the spinal cord in pigs of various ages with myoclonia congenita and in their unaffected littermate controls.

Tissue moisture content determination, lipid extraction, treatment with antioxidant, thin

acid, appears to be too high to assure that the desaturating enzyme is the limiting factor of the reaction, as we have shown in a previous work (1). Consequently, according to our theory that desaturation and esterification compete for the fatty acid, activating effects were very probably expected to be found in these conditions by addition of another fatty acid that would decrease the amount of substrate available for the esterification. Therefore this result and the other reported activating effects of the addition of γ -linolenic acid, eicosa-11,14-dienoic acid or arachidonic acid to the eicosa-8,11,14-trienoic acid system supports our theory (1).

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R. R. BRENNER
Catedra de Bioquimica
Instituto de Fisiologia
Facultad de Ciencias Medicas
La Plata, Argentina

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Tissue moisture content determination, lipid extraction, treatment with antioxidant, thin

TABLE I

Wet Weight, Dry Weight, Moisture Content and Total Lipid Content of Normal and Affected Pig Spinal Cord at Different Ages

Content	Normal					Affected				
	6 days	10 days	14 days	21 days	30 days	6 days	10 days	14 days	21 days	30 days
Tissue wet weight, g	6.04834	3.99112	5.31400	6.50511	6.02575	3.69140	3.05840	4.23584	4.19875	7.25318
Tissue dry weight, g	1.53566	1.87583	1.59473	1.67767	2.17590	0.95644	1.08971	1.14241	1.39958	2.43634
Per cent moisture	74.61	53.00	69.99	74.21	63.89	74.09	64.37	73.03	66.67	66.41
S.D.	8.12	8.05	8.05	8.00	8.05	3.93	3.86	3.86	3.86	3.85
Milligram crude lipid	770.00	975.00	912.50	982.50	1335.00	505.00	587.50	592.50	602.50	1250.00
Per cent crude lipid (of dry tissue)	52.36	53.20	62.15	60.76	64.43	54.38	59.02	56.24	46.64	53.46
No. of determinations	4	4	4	4	5	4	4	4	4	5

layer chromatography (TLC) and phosphorus analysis of the separated phospholipids were carried out as described by Rouser et al. (3). Methyl esters of fatty acids from total lipid extracts were prepared as described by Morrison and Smith (4) using 14% BF_3 in methanol at 100 C for 2 hr. Gas liquid chromatography of the methyl esters was carried out on polar and nonpolar columns with the conditions and standardization procedures for detector response and linearity, and peak identification methods previously described (5,6). Peak areas were determined by electronic integration.

Table I shows that the only consistent difference from normal in the development of spinal cord in affected pigs was a marked decrease in total lipid at all time periods except at 30 days of age although the per cent of crude lipid was unchanged. Samples from affected pigs taken at 30 days had light cases of the disorder. Table II indicates that the decrease of total crude spinal cord lipids in affected pigs noted in Table I is reflected in decreases of each of the phospholipid classes except at 30 days of age. The distribution of phospholipid classes appears to be normal at all ages in affected pigs when results are expressed on the basis of milligram per 100 mg total lipid or as per cent P of total P. TLC in propanol-NH_3/H_2O (6:2:1) revealed no qualitative differences from normal of the pattern of gangliosides present in spinal cord lipids from affected pigs. TLC in CCl_4 indicated that cholesterol esters were absent from the spinal cord lipids of normal and affected pigs at all ages studied. The absence of cholesterol esters from spinal cord lipids of affected pigs suggests that the pathology of this disorder is not associated with demyelination where the presence of cholesterol esters is characteristic (5). Table III shows that total lipid extracts of spinal cord showed an increase in polyenoic acids over normal which was distributed between arachidonic, docosopentaenoic and docosahexaenoic acids, and in linoleic acid at 30 days. The increase was significant at 6 days ($P < 0.001$), 10 days ($P < 0.05$), 14 days ($P < 0.02$), 30 days ($P < 0.01$), but not at 21 days. Oleic acid was noted to decrease at 6 days ($P < 0.05$), 10 days ($P < 0.05$), 14 days ($P < 0.001$), 21 days ($P < 0.02$), but not at 30 days. No marked differences in saturated fatty acids were observed. Increases in fatty acids containing more than 18 carbon atoms in spinal cord lipids of affected pigs were due largely to polyunsaturated fatty acids. The relative increases in polyunsaturated fatty acids were similar to observations made in the lipids of sciatic nerve undergoing Wallerian degeneration after tran-

TABLE II

Phospholipid Composition of Normal and Affected Pig Spinal Cord at Different Ages

Composition	Normal					Affected				
	6 days	10 days	14 days	21 days	30 days	6 days	10 days	14 days	21 days	30 days
Milligram lipid										
PE[a]	137.7	160.9	141.5	137.6	240.3	68.2	85.2	88.9	90.4	225.0
S.D.	19.0	22.2	19.5	19.0	33.6	9.4	11.7	12.3	12.5	30.4
PC[a]	107.8	129.2	109.5	132.6	153.5	68.2	82.3	85.9	75.3	175.0
S.D.	16.9	20.3	17.2	20.8	25.1	10.7	12.9	13.5	11.3	27.5
Sphingomyelin	46.2	56.1	59.3	63.9	73.4	27.8	33.8	38.5	40.7	68.7
S.D.	1.7	2.1	2.3	2.4	2.8	1.1	1.3	1.5	1.6	2.6
PS + PI[a]	61.6	73.1	70.7	59.0	100.1	35.4	48.5	50.4	48.2	93.8
S.D.	1.8	2.1	2.0	1.7	2.9	1.0	1.4	1.5	1.4	2.7
Milligram per 100 mg total lipid										
PE	17.5	16.5	15.5	14.0	18.0	13.5	14.5	15.0	15.0	18.0
S.D.	0.9	0.9	0.8	0.7	0.9	0.7	0.8	0.8	0.8	0.9
PC	14.0	13.3	12.0	13.5	11.5	13.5	14.0	14.5	12.5	14.0
S.D.	1.4	1.3	1.2	1.4	1.2	1.4	1.4	1.5	1.3	1.4
Sphingomyelin	6.0	5.8	6.5	6.5	5.5	5.5	5.8	6.5	6.8	5.5
S.D.	0.2	0.2	0.3	0.3	0.2	0.2	0.2	0.3	0.3	0.2
PS + PI	8.0	7.5	7.8	6.0	7.5	7.0	8.3	8.5	8.0	7.5
S.D.	0.2	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.2	0.2
Per cent P of total P										
PE	38.5	38.4	37.1	35.0	42.4	34.2	34.1	33.7	35.5	40.0
S.D.	2.4	2.4	2.3	2.2	2.7	2.2	2.1	2.1	2.2	2.5
PC	30.8	30.8	28.7	33.8	27.1	34.1	32.9	32.6	29.6	31.1
S.D.	1.8	1.8	1.7	2.0	1.6	2.0	1.9	1.9	1.7	1.8
Sphingomyelin	13.2	13.4	15.6	16.3	12.9	13.9	13.5	14.6	16.0	12.2
S.D.	0.7	0.7	0.8	0.8	0.6	0.7	0.7	0.7	0.8	0.6
PS + PI	17.6	17.4	18.6	15.0	17.7	17.7	19.4	19.1	18.9	16.7
S.D.	0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.3	0.3	0.3

[a]Abbreviations used: PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol.

TABLE III

Fatty Acid Composition of Spinal Cord Lipid Extracts in Normal and Affected Pigs of Various Ages

Fatty acid methyl esters[a]	Normal					Affected				
	6 days	10 days	14 days	21 days	30 days	6 days	10 days	14 days	21 days	30 days
16:0	26.88	25.72	18.09	20.32	18.09	26.87	29.57	22.25	23.77	19.28
S.D.	0.8	0.5	0.1	0.5	0.2	4.5	2.9	5.4	5.4	2.5
16:1	1.16	1.48	1.51	0.78	1.55	2.46	1.59	1.94	1.01	1.00
S.D.	0.3	0.7	0.3	0.2	0.7	0.3	0.8	0.4	0.4	0.4
18:0	9.32	8.55	9.01	11.23	11.08	11.60	9.05	12.41	9.48	8.22
S.D.	0.4	0.1	0.3	0.3	0.3	2.5	2.4	1.1	1.9	2.0
18:1	55.99	59.72	64.01	61.23	64.75	48.02	54.25	52.62	57.28	64.05
S.D.	0.6	0.3	1.8	1.9	2.4	3.9	3.5	1.6	2.3	1.8
18:2	1.30	0.83	1.48	1.34	0.20	1.69	1.66	2.26	1.26	1.59
S.D.	0.4	0.1	0.5	0.3	0.3	0.6	0.7	0.8	0.4	0.3
20:0	0.36	0.11	0.12	0.09	0.10	0.11	0.14	0.35	0.15	0.10
S.D.	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.03	0.03	0.03
20:1	0.68	1.02	1.00	0.62	1.15	0.99	0.71	1.19	0.74	0.88
S.D.	0.3	0.3	0.2	0.2	0.1	0.3	0.2	0.4	0.3	0.3
20:4	0.59	0.85	1.61	1.68	0.75	3.23	1.43	3.34	1.85	1.68
S.D.	0.2	0.4	0.4	0.3	0.2	0.2	0.2	0.4	0.4	0.4
22:5	0.13	0.45	0.63	0.48	0.25	0.84	0.58	1.18	0.70	0.60
S.D.	0.03	0.05	0.02	0.02	0.03	0.2	0.3	0.08	0.07	0.3
22:6	0.15	0.37	0.95	0.85	0.30	1.87	0.78	1.77	1.05	0.94
S.D.	0.04	0.02	0.1	0.1	0.04	0.6	0.08	0.4	0.3	0.3
24:0	0.30	0.09	0.21	0.19	0.21	1.14	0.11	0.12	0.46	0.20
S.D.	0.03	0.01	0.06	0.06	0.04	0.1	0.1	0.02	0.2	0.1
24:1	1.13	0.42	0.62	0.65	1.46	1.16	0.38	0.33	1.12	0.83
S.D.	0.3	0.04	0.06	0.2	0.2	0.3	0.04	0.02	0.5	0.2
Saturated	36.86	34.47	27.43	31.83	29.48	39.72	38.87	35.13	33.86	27.80
Monoenes	58.96	62.64	67.14	63.28	68.91	52.63	56.93	56.08	60.15	66.76
Dienes + Polyenes	2.17	2.50	4.67	4.35	1.50	7.63	4.45	8.55	4.86	4.81
Total $< C_{18}$	94.65	96.30	94.10	94.90	95.67	90.64	96.12	91.48	92.80	94.14
Total $> C_{18}$	3.34	3.31	5.14	4.56	4.22	9.34	4.13	8.28	6.07	5.23
Saturated	0.66	0.20	0.33	0.28	0.31	1.25	0.25	0.47	0.61	0.30
Polyenes	0.87	1.67	3.19	3.21	1.30	5.94	2.79	6.29	3.60	3.22
Monoenes	1.81	1.44	1.62	1.27	2.61	2.15	1.09	1.52	1.86	1.71

[a]Only presented if change noted or if the fatty acid is greater than 1% of the total. Numbers before colon refers to numbers of C atoms. Number after colon represents number of double bonds; 14:0, 20:5, 22:0, 22:1, 23:0, 23:1 present in trace amounts.

section (5) but the relative losses of saturated fatty acids containing more than 18 carbon atoms which were noted in Wallerian degeneration did not occur in spinal cord of pigs with myoclonia congenita.

JAMES F. BERRY
THOMAS F. FLETCHER[1]
MAGDALINE BOVIS
Department of Neurology
University of Minnesota
Health Sciences Center
Minneapolis, Minnesota 55455

[1]Department of Veterinary Anatomy, University of Minnesota, College of Veterinary Medicine, St. Paul, Minnesota 55101.

ACKNOWLEDGMENTS

Supported by U.S. Public Health Service Grants NB-05052-04 and NB-01081 from the National Institute of Neurological Diseases and Strokes.

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[Received April 22, 1969]

The Determination of *Trans* Isomers in GLC Fractions of Unsaturated Esters

ABSTRACT

The determination of the amount of *trans* unsaturated isomers present in collected fractions of unsaturated fatty acid methyl esters separated by gas liquid chromatography is accomplished by measuring the infrared absorbances of the collected peak at 10.36 and 8.55 μ. The ratio of these two absorbances is proportional to the *trans* unsaturation. The interfering polyester column bleed is removed from the collected peaks by collection on alumina and elution into the spectrophotometer cell with CS_2.

INTRODUCTION

The GLC separation of unsaturated fatty esters employing the usual 6-8 ft column of polyester separates the unsaturated esters into fractions according to the number of double bonds in the molecule. However, each unsaturated ester may be a mixture of *cis* and *trans* isomers. Although these geometrical isomers can be separated by capillary columns (1), the positional isomers present in hydrogenated oils interfere with this separation (2).

In partially hydrogenated fats, the *trans*-octadecenoate may be the principal fatty ester present (2) but will be reported as oleate. Also *trans-cis*, and *trans-trans*-octadecadienoate will be included with the *cis-cis*-linoleate. Although the total *trans* isomers may be determined by infrared spectrophotometry (3), this method does not reveal if the *trans* double bond is in a monoene or a diene system.

By a combination of GLC separation and infrared spectrophotometry of the collected fractions of each peak, the isomeric form of each ester can be determined.

The amount of *trans* isomers in a sample of methyl esters is proportional to the ratio of the absorbances at 10.36 μ (*trans* double bonds) and 8.55 μ (ester group) and this ratio is independent of the concentration of the sample (4). Thus, since the ester sample need not be weighed nor made up to known volume, the small samples collected from a gas chromatographic separation may be analyzed.

EXPERIMENTAL PROCEDURES

A Perkin-Elmer Model 21 with a sample micro cell of 0.5 mm path length was used for the spectrophotometric analysis. The instru-

section (5) but the relative losses of saturated fatty acids containing more than 18 carbon atoms which were noted in Wallerian degeneration did not occur in spinal cord of pigs with myoclonia congenita.

JAMES F. BERRY
THOMAS F. FLETCHER[1]
MAGDALINE BOVIS
Department of Neurology
University of Minnesota
Health Sciences Center
Minneapolis, Minnesota 55455

[1]Department of Veterinary Anatomy, University of Minnesota, College of Veterinary Medicine, St. Paul, Minnesota 55101.

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The determination of the amount of *trans* unsaturated isomers present in collected fractions of unsaturated fatty acid methyl esters separated by gas liquid chromatography is accomplished by measuring the infrared absorbances of the collected peak at 10.36 and 8.55 μ. The ratio of these two absorbances is proportional to the *trans* unsaturation. The interfering polyester column bleed is removed from the collected peaks by collection on alumina and elution into the spectrophotometer cell with CS_2.

INTRODUCTION

The GLC separation of unsaturated fatty esters employing the usual 6-8 ft column of polyester separates the unsaturated esters into fractions according to the number of double bonds in the molecule. However, each unsaturated ester may be a mixture of *cis* and *trans* isomers. Although these geometrical isomers can be separated by capillary columns (1), the positional isomers present in hydrogenated oils interfere with this separation (2).

In partially hydrogenated fats, the *trans*-octadecenoate may be the principal fatty ester present (2) but will be reported as oleate. Also *trans-cis*, and *trans-trans*-octadecadienoate will be included with the *cis-cis*-linoleate. Although the total *trans* isomers may be determined by infrared spectrophotometry (3), this method does not reveal if the *trans* double bond is in a monoene or a diene system.

By a combination of GLC separation and infrared spectrophotometry of the collected fractions of each peak, the isomeric form of each ester can be determined.

The amount of *trans* isomers in a sample of methyl esters is proportional to the ratio of the absorbances at 10.36 μ (*trans* double bonds) and 8.55 μ (ester group) and this ratio is independent of the concentration of the sample (4). Thus, since the ester sample need not be weighed nor made up to known volume, the small samples collected from a gas chromatographic separation may be analyzed.

EXPERIMENTAL PROCEDURES

A Perkin-Elmer Model 21 with a sample micro cell of 0.5 mm path length was used for the spectrophotometric analysis. The instru-

TABLE I

GLC Infrared Analysis of a Mixture of Methyl Oleate, Elaidate and Linoleate

Mixture	Known Original sample, %	GLC Separation Found: Octadecenoate fraction, %	GLC Separation Found: Octadecadienoate fraction, %
Methyl oleate	42.1	73.0,73.2,72.8 (73.0)	
Methyl elaidate	31.5		
Methyl linoleate	26.4		27.0,26.8,27.2 (27.0)
% *Trans*[a]		42.4,41.4,41.9 (41.9)	1.0,2.1,2.2 (1.8)

[a]From IR analysis of GLC fractions % *trans* = .419(73.0) + .018(27.0) = 31.0%.

ment was adjusted to read 0 absorbance at 10.36 μ with carbon disulfide in both sample and reference cells and to cover 100% span so total absorbance could be recorded.

Gas liquid chromatographic separations were made using an Aerograph Model A90P with a ¼ in. x 12 ft column packed with 15% diethylene glycol succinate on Chromosorb W, 60/80 mesh. The column was held at 212 C with 70 ml/min He.

There is a continuous bleed of the polyester liquid phase from the chromatograph column that is mixed with the collected ester fractions. This material has an infrared absorption peak at about 8.6 μ that interferes with the absorbance ratio of the fatty ester so it must be removed from the samples. We found that polyester bleed material was adsorbed on alumina and was not eluted by carbon disulfide. Therefore the GLC fractions were collected in 1 x 100 mm melting point capillary tubes that had a 3 to 4 mm section packed with 60-80 mesh alumina held in the central part of the tube with glass wool.

When a fraction was eluted from the gas chromatograph, the collecting tube was inserted into the outlet and the fraction collected. The collecting tube was not cooled and no fog was produced. After the fraction was collected, the ester was washed through the alumina into the microcell with 10-20 μl of carbon disulfide and the absorbance values of the peaks at 8.55 and 10.36 μ recorded. The sample was diluted if the absorbance was over 0.8. The ratio of the two absorbances, $A_{10.36}/A_{8.55}$, is calculated and substituted into the linear equation, % *trans* = K $(A_{10.36}/A_{8.55})$ - k. The values of the two constants K k, are found from the absorbance ratios of known samples. K is the slope of the line relating % *trans* and absorbance ratio and k is the y intercept. By analysis of known mixtures of methyl oleate and methyl elaidate (high purity esters obtained from the Hormel Institute, Austin, Minn.) in carbon disulfide, we found the equation to be:

$$\% \text{ Methyl elaidate} = 121.9\,(A_{10.36}/A_{8.55}) - 9.2$$

RESULTS AND DISCUSSION

The results of a GLC Infrared Analysis of a mixture of methyl oleate, elaidate and linoleate are shown in Table I. As shown, the *trans* isomers were correctly located as part of the octadecenoate from the percentage of that fraction that is *trans*. The method described shows good accuracy and precision.

Carbon disulfide was used as a solvent although other solvents, such as chloroform, could be used. However, the constants in the equation depend on the solvent. For example, in chloroform the value of K in the equation was found to be 181.0.

This infrared method of analysis is believed to be a general method for mixtures of isomers whose components have several infrared absorptions in common but one that is peculiar to one isomer. It is quite rapid since the samples need not be weighed or made up to a known concentration.

ROBERT R. ALLEN
Anderson Clayton Foods
3333 N. Central Expressway
Richardson, Texas 75080

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[Received June 9, 1969]

Oxidation of α-Tocopherol to α-Tocopheryl Quinone by Carbon Tetrachloride-Ethanol Solvent

ABSTRACT

The addition of small amounts of CCl_4 to ethanolic solutions of α-tocopherol, vitamin A acetate or β-carotene caused destruction of these fat-soluble compounds. The oxidation product of α-tocopherol was identified as α-tocopheryl quinone by UV and IR spectral analysis. Maximum conversion to the quinone occurred with a ratio of CCl_4 to ethanol of 25:75 (v/v).

Butler (1) has shown that CCl_4 induced the non-enzymatic oxidation of reduced glutathione, cysteine and cytochrome C at physiological temperatures. Hove (2) reported that CCl_4 catalyzed the bleaching of β-carotene in ethanol solution in the presence of linoleate hydroperoxide. Recknagel and Ghoshal (3) found that a conjugated diene was formed from the linoleic acid of liver microsomes after CCl_4 treatment. Butler (1) has shown that CCl_4-induced oxidations resulted in an inverse substitution of one of the chlorine atoms, presumably through free radical formation, and assumed that the final fate of this chlorine atom was as a chloride ion produced by acquisition of two electrons from the substrate compound. He suggested that this oxidative activity may be involved in the hepatotoxic effect of CCl_4 in animals. If this is true, then antioxidants should confer protection against the toxic action of this solvent. Indeed, several years ago Hove (4) did show that α-tocopherol and other antioxidants had a marked protective effect against fatal CCl_4 poisoning of rats fed low protein diets. This finding has recently been confirmed by Gallagher (5) and by Seward et al. (6). Therefore it is of interest to find now that CCl_4 can oxidatively destroy α-tocopherol by causing its conversion to the quinone.

When glass-distilled spectral grade CCl_4 was added to ethanolic solutions of dl-α-tocopherol (70 μg/ml), conversion to the tocopheryl quinone began quickly and proceeded to near completion within a period of several days (Fig. 1). Quantitative conversion, as observed spectrophotometrically, occurred within 20 days when the CCl_4 concentration was 20% (v/v). In control solutions (no CCl_4) under the same time and conditions, no change occurred. The loss of the tocopherol was indicated by the complete disappearance of the Emmerie-Engel reaction and by loss of the UV absorption peak at 293 mμ (Fig. 2). The UV curve showed the strong bicuspid peaks at 264 and 273 mμ that are characteristic of the quinone, with $E^{1\%}_{1cm}$ (273)=345 (Fig. 3). The IR spectrum of the dry product conformed exactly to that of authentic α-tocopheryl quinone (7). In another trial (Fig. 1 and 2), $CHCl_3$ was found to be less than half as active as CCl_4 in oxidizing tocopherol to the quinone.

The rapidity and degree of oxidation of tocopherol by CCl_4 depends on the relative ratio of the two solvents, ethanol and CCl_4. In either pure CCl_4 or pure ethanol, tocopherol was quite stable. The maximum rate of oxidation occurred with 25% CCl_4 in ethanol

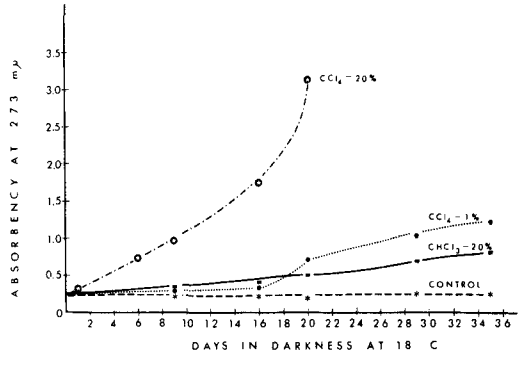

FIG. 1. UV curves showing the production of α-tocopheryl quinone (273 mμ) from α-tocopherol, induced by low levels of CCl_4 at 18 C. α-Tocopheryl quinone production was monitored by periodic UV readings over 36 days.

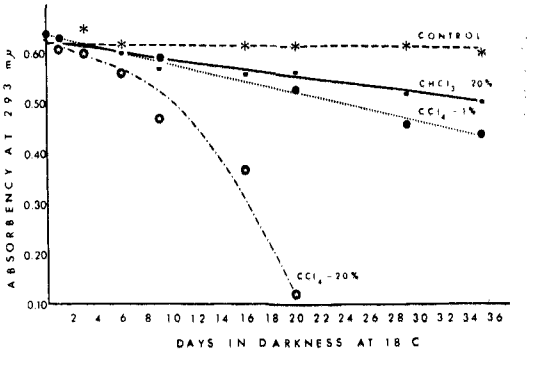

FIG. 2. UV curves showing the loss of absorption at 293 mμ (α-tocopherol) induced by low levels of CCl_4 at 18 C. α-Tocopherol loss was monitored by periodic UV readings over 36 days.

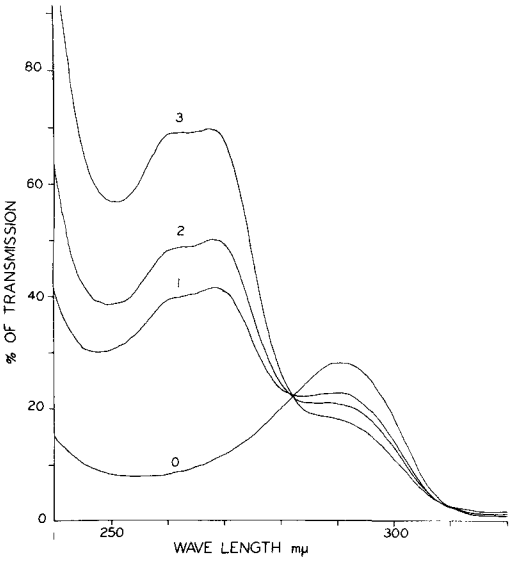

FIG. 3. UV curves showing the concomitant loss of α-tocopherol (294 mμ) and production of α-tocopheryl quinone (264, 273 mμ) induced by low levels of CCl_4 at 18 C. Volumes of CCl_4 for curves 0, 1, 2 and 3 were 0%, 1.25%, 2.50% and 5.00% in ethanol, respectively.

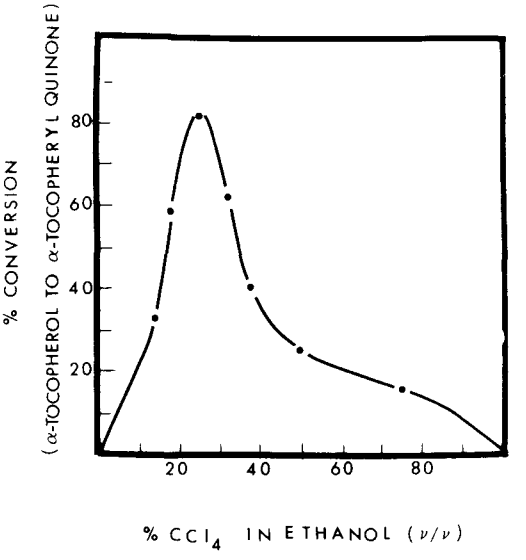

FIG. 4. Effect of proportions of CCl_4 in ethanol on the oxidation of α-tocopherol to α-tocopheryl quinone, at 18 C over a 4 day period in dim, diffuse, laboratory light.

(v/v), as shown in Figure 4.

We have obtained other data showing that vitamin A acetate is even more labile to the solvent effect than is tocopherol. As little as 1.3% of CCl_4 added to vitamin A acetate in a mixture of ethanol and xylene (11 μg/ml) caused the destruction of 53% in 4 days; control solutions with no CCl_4 were completely stable under identical conditions.

The oxidative effect of CCl_4 probably depends on free radical primers. Polar compounds such as ethanol could be such primers, and could explain the effect of the mixed solvents. We also found that diffuse laboratory light was a primer; in 4 days the destruction of β-carotene by 5% CCl_4 was 85% in light but was only 30% in total darkness. In the control solutions (without CCl_4) no loss occurred in this period, even in the light. Short wavelength UV light (254 mμ) has been found to be an especially effective primer. Under this radiation, the reaction time was minutes rather than days, but approximately the same amount of destruction occurred. We have also noted that a high level of hydroquinone (0.1 g/4.2 ml reaction mixture) almost completely inhibited the CCl_4-induced oxidations.

The oxidative potential of CCl_4 under certain conditions may have a relation to its hepatotoxic action, as has been suggested in the literature. In addition, this property has important implications for the extraction of samples and storage of samples and standards in analytical procedures for easily oxidized fats and fat-soluble components.

C. R. SEWARD
G. VAUGHAN MITCHELL
L. C. ARGRETT
E. L. HOVE
Division of Nutrition
Food and Drug Administration
Department of Health, Education and Welfare
Washington, D. C. 20204

ACKNOWLEDGMENT

E. P. White of the Ruakura Agricultural Research Centre in Hamilton, New Zealand confirmed the interpretation of the IR curves of the experimental quinone prepared by E. L. Hove while on a fellowship at that institution.

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[Received April 1, 1969]

Erratum

In the paper entitled "Fatty Acid Desaturase Systems of Hen Liver and Their Inhibition by Cyclopropene Fatty Acids" by A. R. Johnson et al., which appeared in Lipids, Vol. 4, No. 4, July 1969, the legend of Figure 2 should belong to Figure 1 and the legend of Figure 1 should belong to Figure 2. See correct placement below.

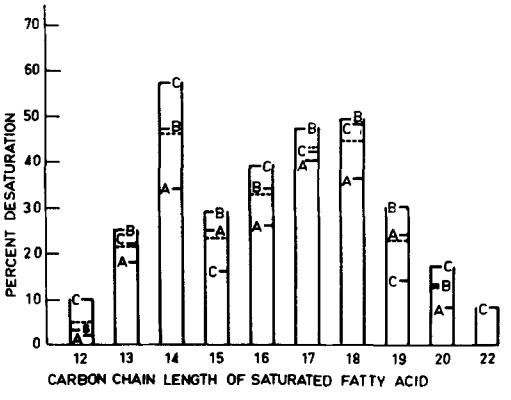

FIG. 1. Desaturation of fatty acids of varying chain length by the desaturase system prepared from each of three hen livers, A, B and C. Dotted line represents mean; least significant difference between means (P = 0.05) = 10.1%.

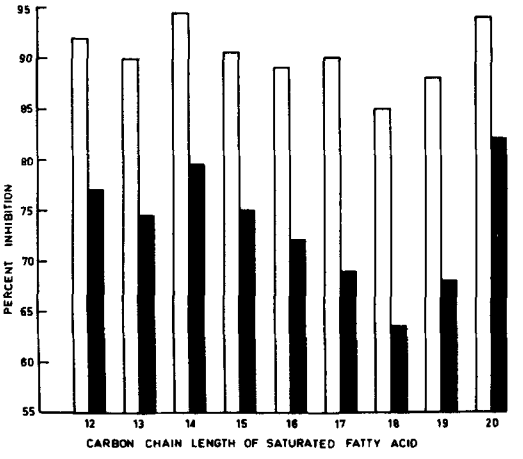

FIG. 2. Mean per cent inhibition of desaturation of fatty acids of varying chain lengths by sterculic and malvalic acids (0.0005 mM) in desaturating systems from two hen livers (A and B, Fig. 1). The open blocks represent sterculic acid and shaded blocks malvalic acid. The per cent inhibition was calculated using the per cent desaturation from the uninhibited systems as 100% conversion.