Circadian Rhythm of Fatty Acid Desaturation in Mouse Liver

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ABSTRACT

A study was made of the diurnal changes in liver microsomal desaturation of labeled stearic, linoleic and α-linolenic acids to oleic, γ-linolenic and octadeca-6,9,12,15-tetraenoic acids, respectively. C3H-S mice were used and were exposed to light-dark cycles. A circadian rhythm was observed for stearic acid desaturation, and a different one for linoleic acid. Linoleic and α-linolenic desaturation had similar responses in the day cycle. This would indicate that different mechanisms control the oxidative desaturations of the fatty acids in the 9 and 6 carbons. The fatty acid composition of the whole liver and liver microsomes also showed variations. Remarkable oscillations were observed for stearic and oleic acids. Neither the total protein synthesis nor the free fatty acid concentration in the microsomes followed a rhythm parallel to the desaturation of the studied fatty acids. The injection of cycloheximide 4 hr before measuring the desaturation modified the circadian variation of both the 9 and 6 desaturations. The modification induced by cycloheximide was considered to indicate that both variations are related to the synthesis of specific proteins but not to that of a degradative or inhibitory protein.

Fatty acid olefination is one of the fundamental reactions in the biosynthesis of unsaturated fatty acids in the animal and is produced by an oxidative desaturation. The substrate of the reaction is acyl-CoA. The enzymes that take part in it are linked to the endoplasmic reticulum and catalyze the formation of a double bond in saturated and unsaturated fatty acids in the presence of NADH and 0_2 (1). Apparently there are various desaturases, and the information gathered up to the present indicates that the same enzyme desaturates oleic, linoleic and α-linolenic acid, while stearic acid is desaturated by a different one (2). These enzymes are called, respectively, 6 desaturase and 9 desaturase, according to the position in which the new double bond is formed (3).

Several factors influence the oxidative desaturation, contributing to the regulation of the production of unsaturated fatty acids. (2).

In the present work, and in order to contribute to the clarification of the regulation mechanisms, studies were made on the circadian rhythm of the oxidative desaturation of stearic, linoleic and α-linolenic acids by mouse liver microsomes. At the same time, the daily variations of the microsomal free fatty acid (FFA) content, the composition of the fatty acids of whole liver and liver microsomes, the synthesis of liver proteins and the effect of inhibitors on the protein synthesis, were also investigated.

METHODS

Treatment of Animals

Female C3H-S mice of 6 weeks of age were used. They were maintained since birth at 25 C ± 1 C on water and Purina chow ad libitum, and exposed to illumination (fluorescent white light 40 W) from 06:00 to 18:00 hr alternating with 12 hr of darkness. The fatty acid composition of the diet was: 2.6% myristic acid; 19.4%

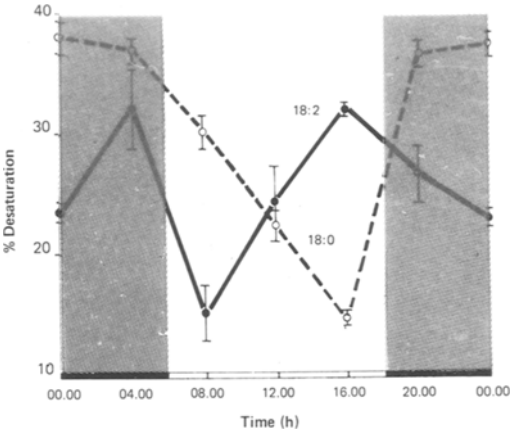

FIG. 1. Circadian changes of the desaturation of linoleic acid to γ-linolenic acid and stearic to oleic acid. Curve of 18:0/P between 00:00 hr vs. 16:00 hr <0.001; curve of 18:2/P between 04:00 hr vs. 08:00 hr $<0.005>0.001$; between 08:00 hr vs. 16:00 hr <0.001; between 16:00 hr vs. 00:00 hr <0.001.

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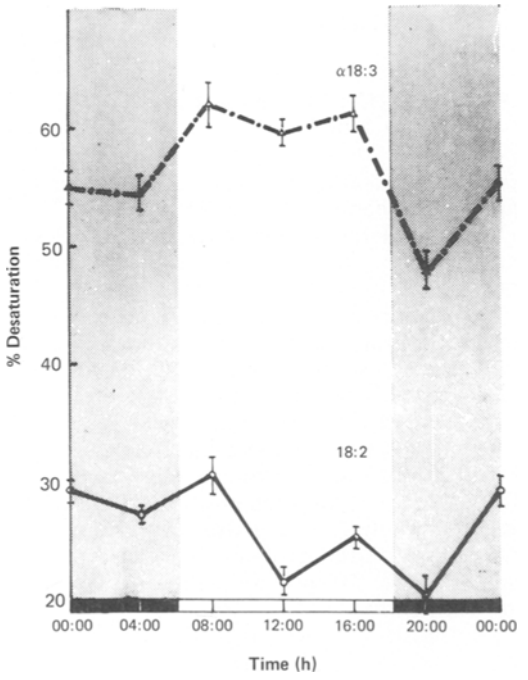

FIG. 2. Circadian change of linoleic and α-linolenic 6 desaturation. Curve of 18:2/P between 08:00 hr vs. 12:00 hr <0.001; between 00:00 hr vs. 20:00 hr <0.001; between 12:00 hr vs. 16:00 hr <0.001; between 16:00 hr vs. 20:00 hr $<0.005>0.001$; curve of α-18:3/P between 08:00 hr, 12:00 hr and 16:00 hr vs. 20:00 hr <0.001.

palmitic acid; 2.7% palmitoleic acid; 3.2% stearic acid; 26.7% oleic acid; 45.4% linoleic acid; and traces of α-linolenic acid.

All the experiments were performed according to a transversal design (4). The animals were killed at 4 hr intervals during a 24 hr period, and each experiment did not last more than a week. To measure the fatty acid-desaturating capacity of liver microsomes, four pools of two mice each were used each time. To study the microsomal FFA content, the composition of the fatty acids of whole liver and liver microsomes, and the incorporation of leucine-C^{14} in liver proteins, six animals were analyzed independently. When the effect of cycloheximide on the desaturation of fatty acids was studied, male mice of 4 months of age were used under the same conditions. Four pools of two normal animals each were killed at 4 hr intervals. Likewise, 4 similar pools were intraperitoneally injected 4 hr before being killed with 0.5 mg cycloheximide in physiological solution per 100 g of weight.

Determination of Per Cent Desaturation

Stearic, linoleic and α-linolenic acids, labeled with ^{14}C in the carboxyl carbon were used.

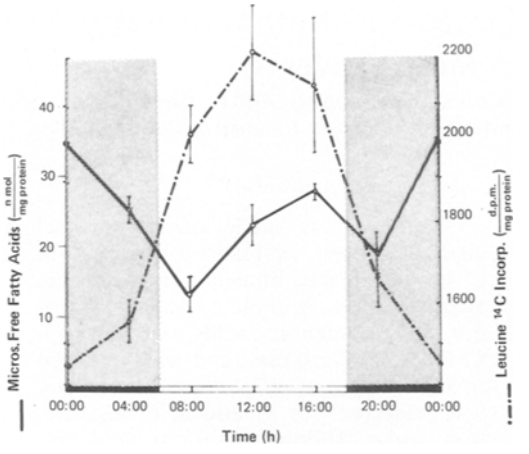

FIG. 3. Diurnal oscillations of leucine-1 ^{14}C incorporation in the liver proteins and FFA content of liver microsomes. Curve of leucine-1 ^{14}C incorporation: P between 00:00 hr vs. 12:00 hr <0.001. Curve of FFA content: P between 00:00 hr vs. 08:00 hr $<0.005>0.001$; between 08:00 hr vs. 16:00 hr <0.001.

They were purchased from The Radiochemical Centre, Amersham, England, and were 98% radiochemically pure. The acids were diluted with the same unlabeled acids (Hormel Institute, Austin, Minn.) to obtain a specific activity of 7.26 μC/μmol, 5.60 μC/μmol and 4.15 μC/μmol respectively.

After killing the animals, the livers were immediately excised and cooled at 0 C. They were homogenzied in the cold with a solution of 0.15 M KCl, 5 mM $MgCl_2$, 1.5 mM glutathione, 62 mM phosphate buffer (pH 7.0) and 0.25 M sucrose in 0.1 mM EDTA 3:1 v/w. The homogenate was centrifuged at 10,000 x g for 30 min, and the supernatant was recentrifuged at 110,000 x g for 1 hr. The pellet was resuspended in homogenizing solution (2:1 v/v) and proteins estimated by the buret method (5).

The desaturating capacity of the liver microsomes was determined by incubation of 10 nmol labeled acid at 35 C and pH 6.8 in air with 5 mg microsomal protein during 20 min. The incubation medium contained in μmol: ATP, 2.5; CoA, 0.2; NADH, 2.5; $MgCl_2$, 15: glutathione, 4.5; nicotinamide, 1; NaF, 125; phosphate buffer (pH 7.0), 125; in 0.15 M KCl and 0.25 M sucrose. The total volume was 3 ml. The 10 nmol acid were dissolved in 10 μl propyleneglycol. The incubation was stopped by the addition of 2 ml alcoholic 10% KOH. After saponification at 80 C for 45 min, the fatty acids were extracted and esterified with methanolic 3 N HCl for 3 hr at 63 C. The per cent of fatty acid desaturated was measured by

gas liquid radiochromatography in a Pye apparatus with proportional counter, using 10% diethylene-glycolsuccinate on Chromosorb W (80-100 mesh) at 180 C, as has been described previously (6). All determinations were performed in duplicate.

Incorporation of 1-^{14}C-Leucine in Liver Proteins

The animals received an intraperitoneal injection of 0.2 ml DL-leucine-1 ^{14}C in physiological solution (25 mC/mM) in a dosage of 10 μC/mouse 1 hr before being killed. Each of the excised livers was homogenized with 0.25 M sucrose 3:1 v/w. In an aliquot of the homogenate, proteins were precipitated with 10% trichloroacetic acid v/v. The precipitate was washed twice with 5% trichloroacetic acid v/v and then twice with 96% ethanol. It was stored overnight at 37 C and then dissolved in 1 N KOH. The concentration of proteins was determined by the buret method (5). Radioactivity was measured in a Packard scintillation counter. The results were expressed in dpm/mg protein.

Evaluation of FFA in Liver Microsomes

The livers were homogenized in 0.25 M sucrose 3:1 v/w. The microsomes were separated by differential centrifugation by the method already described. Each one of the pellets was resuspended in 2 ml 0.25 M sucrose, and the FFA were estimated in aliquots of 400 μl by the method of Dole and Meinertz (7). Then 2.5 ml of the Dole mixture (isopropanol-hexane-1 N H_2SO_4 4:1:0.1 v/v/v) was added, and after shaking, 1.5 ml hexane and 1 ml H_2O were added. After shaking and decanting, the upper phase was separated and an aliquot of it was titrated with 0.1 N NaOH. The results are expressed in nmol/mg protein.

Composition of Fatty Acids of Whole Liver and Liver Microsomes

Both 50 mg of the whole liver and 50 mg of microsomes of the previous experiment were separated and saponified in 10% KOH at 80 C during 45 min under N_2. After acidification, the total fatty acids were extracted with petroleum ether. After evaporation to dryness, they were esterified with methanolic 3N HCl during 3 hr at 63 C. The methyl esters were analyzed by gas liquid chromatography on diethylene-glycol-succinate on Chromosorb W (80-100 mesh) at 174 C, in a Pye chromatograph with an argon ionization detector.

Statistical Analysis

In all the figures the results are expressed as the mean ± standard error. The analysis of statistical significance of the results was made by Student's *t* test.

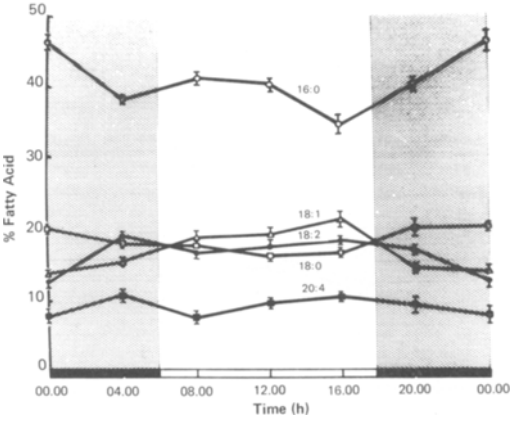

FIG. 4. Circadian changes of the fatty acid composition of the whole liver. Curve of 16:0/P between 00:00 hr vs. 16:00 hr <0.001. Curve of 18:0/P between 00:00 hr vs. 12:00 hr <0.001. Curve of 18:1 : P between 00:00 hr vs. 16:00 hr <0.001. Curve of 18:2/P between 00:00 hr vs. 04:00 hr <0.001. Curve of 20:4/P between 04:00 hr vs. 08:00 hr <0.01.

RESULTS

The changes in the microsomal desaturation of stearic to oleic acid and of linoleic to γ-linolenic acid in the course of the day are shown in Figure 1. In this figure the dark and light are indicated. As has been demonstrated repeatedly (8), the period of activity of mice is at night and they feed preferentially during this period, resting during the day. It can be observed that both studied acids undergo circadian rhythms in the desaturation. The stearic acid curve exhibits its maximal peak (38% desaturation) in the dark period (between 20:00 and 04:00 hr) and the minimum (15.5% desaturation) at the end of the light period. The linoleic acid curve, on the other hand, shows two maxima with 32% desaturation at different times than the stearic (at 04:00 and 16:00 hr) and minima at 08:00 and 00:00 hr with 15% and 23% desaturation, respectively.

The following experiment was to study simultaneously the diurnal changes in the desaturation of linoleic and α-linolenic acids. Figure 2 illustrates that the desaturation of α-linolenic acid to octadeca-6,9,12,15-tetraenoic acid also undergoes circadian oscillations. α-Linolenic acid showed a higher desaturation than linoleic acid in the whole day cycle. However both cycles were rather similar. While the first experiment was done in winter, this experiment was done in summer. Therefore, although a general pattern of the rhythm of linoleic acid desaturation was similar to that shown in Figure 1, there was a displacement in the position of the maxima and minima of the

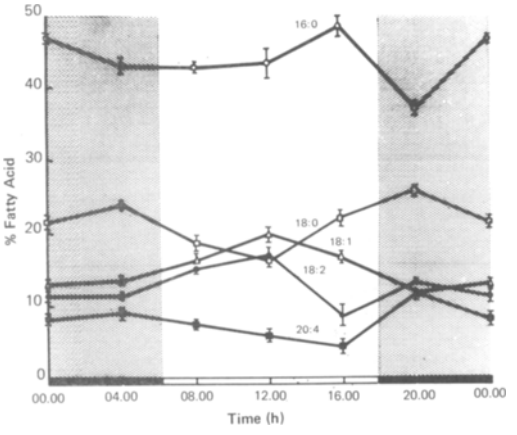

FIG. 5. Circadian changes of the fatty acid composition of liver microsomes. Curve of 16:0/P between 16:00 hr vs. 20:00 hr <0.001; curve of 18:0/P between 12:00 vs. 20:00 hr <0.001. Curve of 18:1/P between 00:00 hr vs. 12:00 hr <0.001; curve of 18:2/P between 12:00 hr vs. 16:00 hr $<0.010>0.005$; curve of 20:4/P between 16:00 hr vs. 20:00 hr <0.001.

curve. The existence of seasonal influences on circadian rhythms has already been described by other authors (9,10).

There may be several causes of a circadian rhythm in fatty acid desaturation that can be related to variations of enzymatic acitivy or changes in the microsomal composition, or both. Several experiments were performed to investigate this possible mechanism.

Figure 3 shows the incorporation of leucine-1-^{14}C in liver proteins in the course of the day. It may be considered that the curve presents the variation of the synthesis of liver total proteins. The incorporation of the radioactive amino acid changes according to the hour of the day in which the experiment is performed, showing a clear circadian rhythm. The maximal values are reached between 08:00 and 16:00 hr, during the light period that corresponds to the resting time of the animals. The minimum is at 00:00, during the period of active feeding. These results agree with those found by Rebolledo and Gagliardino (9) in muscle of mice of the same strain and age, but does not correlate with the curves of fatty acid desaturation.

Figure 3 also exhibits the changes of the microsomal FFA content in the course of the day. The FFA content increases during the dark period, reaching its maximal value at 00:00 and the mimimum at 08:00 hr. Then it begins to rise again during the light period up to 16:00 hr, when a new decrease begins. Nevertheless this curve is not correlated to those of desaturation (Fig. 2).

The changes in the fatty acid composition of the whole liver and liver microsomes in the course of the day are shown in Figures 4 and 5. This experiment was simultaneous with that described in Figure 3. For the sake of accuracy, only the most abundant acids were measured: palmitic, stearic, oleic, linoleic and arachidonic. The curves of Figures 4 and 5 illustrate rather similar variations in the fatty acid composition of whole liver and microsomes, although the oscillations are more pronounced in microsomes. Rather important and opposite changes for stearic and oleic acids are found. The stearic acid maximum is found in the dark period, whereas oleic acid is higher in the light period. In the whole liver there is a correlation between linoleic acid and arachidonic acid that is only partial in the microsomes.

The next step was to investigate the influence of cycloheximide--an inhibitor of protein synthesis--on the diurnal variations of the desaturation of stearic acid and α-linolenic acid. The animals used in this experiment were of a different age than those used in the other experiments, as indicated in Methods. Figure 6 shows the results for α-linolenic acid desaturation. In this experiment the desaturation curve for normal animals does not exhibit a very clear rhythm. In spite of this, the effect of cycloheximide at different moments of the day can be observed. In the rising parts of the curve, the cycloheximide diminishes or nullifies the increase. In the descending parts, the effect is seen in an accentuation of the decay. In Figure 7 the effect of cycloheximide in the desaturation of stearic acid proves to be similar, except for the increase produced at 00:00 hr, which is not inhibited by the cycloheximide.

DISCUSSION

Several works published during the last years have demonstrated the existence of circadian changes related to different biological processes (11,12). Within the lipid field, Shapiro and Rodwell (13) and Hickman et al. (14) have studied the diurnal variation of the hepatic synthesis of cholesterol. Scott and Potter (15) have demonstrated that circadian rhythms are produced in the incorporation of CH_3COO^- into CO_2 and in lipids of different rat tissues. The changes in the lipogenesis in the course of the day were investigated by Kimura et al. (16).

The results of the present work (Figures 1 and 2) demonstrate that the oxidative desaturation of the stearic, linoleic and α-linolenic fatty acids is also modified in the course of the 24 hr of the day. The data were obtained with low substrate concentration. Consequently the de-

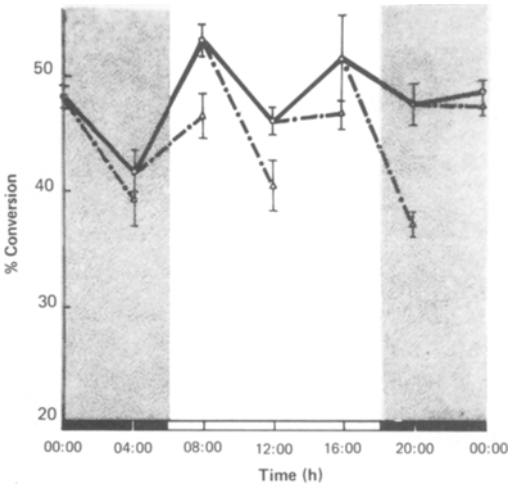

FIG. 6. Effect of cycloheximide on α-linolenic desaturation. o——o Normal animals; △—•—△ animals injected with cycloheximide.

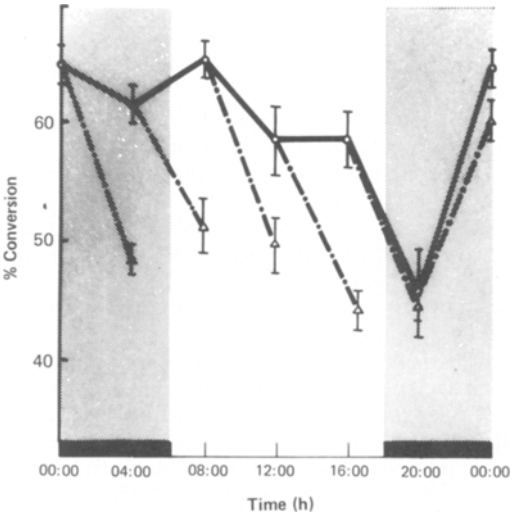

FIG. 7. Effect of cycloheximide on stearic acid desaturation. o——o Normal animals; △—•—△ animals injected with cycloheximide.

saturating capacity of the cellular endoplasmic reticulum was measured, but not necessarily the enzymatic activity. Besides, since the oxidative desaturation of linoleic acid and α-linolenic acid constitutes the first step in the synthesis of the fatty acids of the corresponding families (2,17) and is probably the critical step in the sequence of reactions (18-19), the results could be correlated with a circadian variation of the liver biosynthesis of the respective polyenoic fatty acids at the microsomal level.

As shown in Figures 4 and 5, the fatty acid compositions of whole liver and liver microsomes are also changed in the course of the day. However no apparent correlation was observed when comparing, for example, the oxidative desaturation of linoleic acid (Fig. 2) with the per cent of linoleic and arachidonic acids in liver (Figs. 4 and 5). Both curves of desaturation and fatty acid composition are apparently a consequence of the feeding rhythm of the animals that, besides the incorporation of dietary components, provokes a general rhythmic variation in hormonal secretion and enzymatic activity. They agree with those of Scott and Potter (15), who pointed out that the diurnal variations of lipogenesis depend mainly on the feeding period of the animals. Besides, the effect of different diets and fasting on fatty acid desaturation has already been proved (3,20,21).

The acceptable resemblance between the rhythms of linoleic and α-linolenic acid desaturation (Fig. 2) would also agree with the results that demonstrate that both acids are desaturated by the same enzyme, the 6 desaturase (2). Likewise, the remarkable difference between the curves of stearic and linoleic acid desaturation (Fig. 1) would confirm the existence of two different enzymes, 9 desaturase and 6 desaturase (2,3) regulated by at least partially different mechanisms.

The mechanism that evokes the diurnal change in the fatty acid oxidative desaturation is difficult to discern. The desaturation of fatty acids may be modified by competition with other fatty acids of the same or different series, by hormonal effects probably through enzymatic induction and by changes of related metabolites and cofactor concentrations (2). Therefore a possible cyclical change in the amount of endogenous microsomal FFA competing with the desaturation of the assayed substrate could produce an apparent circadian rhythm of the oxidative desaturation of this substrate.

In Figure 3 there is a clear evidence of the existence of circadian variations in the FFA content of liver microsomes. They may be correlated to the feeding of the animals, since the greatest increase is produced during the first part of the dark period, in which they feed actively. Besides, the peak observed at 16:00 hr is probably related to the nonesterified fatty acids released by the adipose tissue during the light period of rest and fasting (22). However the curve of Figure 3 is apparently not correlated with any of the desaturation curves. Consequently the microsomal FFA concentration does not seem to be a direct cause of the circadian changes of the desaturation. Nevertheless, if the activity of the desaturating enzyme is really related in some way to the concentra-

tion of liver FFA, the results obtained indicate that its effect would be complemented by other factors that make both cruves (FFA and desaturation) noncorrelative.

According to the results in Figure 3, the protein synthesis does not follow the same rhythm as either stearic or linoleic acid desaturation. This indicates that, if the changes observed in the desaturation depend on the synthesis of the corresponding enzymes, the latter may have a particular rhythm that does not exactly correspond to that of the circadian changes of total proteins.

The experiment with cycloheximide provides more information on the relationship between protein synthesis and desaturation. In the desaturation of α-linolenic acid, the previous injection of cycloheximide produces a decrease in the desaturation levels (Fig. 6). Considering that cycloheximide inhibits protein synthesis, these results would indicate that the changes of desaturation are somehow dependent on the synthesis of the related enzymes. Besides, the fact that cycloheximide does not prevent the decay in the desaturation, and even accelerates it, is interpreted, by Shapiro and Rodwell (13) and other authors (23,24) in similar situations, to mean that the variations of the desaturation are not affected by the synthesis of a degradative or inhibitory protein of the enzymes. For stearic acid the interpretation may be similar. However the increase produced at 00:00 hr is not inhibited by cyclohexamide and consequently is probably not related to enzyme synthesis.

The enzymes responsible for the observed changes in the fatty acid desaturation may be specifically the desaturase, acyl-CoA synthetase or even other enzymes that may compete with the desaturase. However the contribution of the acyl-CoA synthetase may be discarded, since Lippel (25) has shown that acyl-CoA synthetases are unaffected by fasting and has suggested that they are not adaptive enzymes.

Therefore the present experiments suggest that the fatty acid desaturation follows circadian changes related to the food intake and governed by protein synthesis and other factors. Moreover circadian changes are also found in the liver fatty acid composition, microsomal FFA content and protein synthesis. These circadian changes must be taken into consideration when comparative studies are made on fatty acid metabolism.

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REFERENCES

1. Nugteren, D.H., Biochim. Biophys. Acta 60:656 (1962).
2. Brenner, R.R., Lipids 6:567 (1971).
3. Inkpen, C.A., R.A. Harris and F.W. Quackenbush, J. Lipid Res. 10:277 (1969).
4. McHugh, R.B., in "The Cellular Aspects of Biorhythms," Edited by H. von Mayersbach, Springer Verlag. Berlin/Heidelberg, 1967, p. 61.
5. Cornall, A.G., G.J. Bardawill and M.M. David. J. Biol. Chem. 177:751 (1949).
6. Brenner, R.R., and R.O. Peluffo, Ibid. 241:5213 (1966).
7. Dole, V.P., and H. Meinertz, Ibid. 235:2595 (1960).
8. Nash, R.E. de P., and E. Llanos. Rev. Soc. Argent. Biol. 45:181 (1969).
9. Rebolledo, O.R., and J.J. Gagliardino, J. Interdiscipl. Cycle Res. 2:101 (1971).
10. von Mayersbach, H., in "The Cellular Aspects of Biorhythms," Edited by H. von Mayersbach, Springer-Verlag, Berlin/Heidelberg, 1967, p. 87.
11. Eling, W., Ibid., p. 105.
12. Wurtman, R.J., and J. Axelrod, Proc. Nat. Acad. Sci. (Washington, D.C.) 57:1594 (1967).
13. Shapiro, D.J., and V.W. Rodwell, Biochem. Biophys. Res. Commun. 37:867 (1969).
14. Hickman, P.E., B.I. Horton and J.R. Sabine, J. Lipid Res. 13:17 (1972).
15. Scott, D.F., and V.R. Potter. Fed. Proc. 29:1553 (1970).
16. Kimura, T., T. Maji and K. Ashida, J. Nutr. 100:691 (1970).
17. Marcel, J.L., K. Christiansen and R. Holman, Biochim. Biophys. Acta 164:25 (1968).
18. Ullman, D., and H. Sprecher, Ibid. 61:70 (1971).
19. Castuma, J.C., A. Catalá and R.R. Brenner. J. Lipid Res., In press.
20. Gómez Dumm, I.N.T. de, M.J.T. de Alaniz and R.R. Brenner, Ibid. 11:96 (1970).
21. Brenner, R.R., R.O. Peluffo, O. Mercuri and M.A. Restelli, Amer. J. Physiol. 215:63 (1968).
22. Scow, R.O., and S.S. Chernick, In "Compresensive Biochemistry," Vol. 18, Edited by M. Florkin and E.H. Stotz, Elsevier Publishing Co., Amsterdam, 1970, p. 19.
23. Kenney, F.T., Science 156:525 (1967).
24. Levitman, I.B., and T.E. Webb, Fed. Proc. 28:729 (1969).
25. Lippel, K., Biochim. Biophys. Acta 239:384 (1971).

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Ascarosides of the Ovaries and Eggs of *Ascaris lumbricoides* (Nematoda)

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ABSTRACT

Nearly all ascarosides in the ovaries of *A. lumbricoides* occurred as esters. Among 15 identified esters, 13 occurred at concentrations exceeding 0.1% of the total ascarosides. Each ester consisted of one or two moles of glycone (ascarylose) and several homologs of long chain aglycone. The acid residues consisted of 95.6 mol % acetate and 4.4 mol % propionate. When present, propionate occurred only at the 2' position of the glycone, which was always esterified. Esters with more than one free hydroxyl group were scarce, 89 wt % of the ascarosides being completely esterified. Monol aglycones contained 25-32 carbon atoms. The C_{29} homolog was most abundant, and ca. 25 wt % of these aglycones were branched and even-numbered. Diol aglycones were unbranched C_{29-35} homologs, C_{31} and C_{33} being about equally abundant in diol ascarosides and C_{33} most common in diol diascarosides. Within each ascaroside class the proportions of different homologs were correlated with the extent of esterification. Free ascarosides of the ascaroside layer of the egg shell, which are formed from the ovarian esters, consisted of 12% monol ascaroside, 10% diol ascaroside, 8% diol diascaroside and 70% diol ascaroside containing an acetylated aglycone. The absence from ascaroside esters of the α-methyl butyrate and α-methyl valerate residues which are plentiful in ovarian triglycerides and waxes suggests considerable biosynthetic specificity.

INTRODUCTION

Lipids from the ovaries and egg shells of *Ascaris lumbricoides* and *Parascaris equorum* (= *A. megalocephala*) contain large amounts of high molecular weight glycolipids known as ascarosides (1,2). The unsaponifiables of *P. equorum* adults, which live in the intestine of the horse, were found to contain three related ascarosides (Fig. 1) in which one or two moles of the glycone (ascarylose; 3.6-dideoxy-L-arabinohexose) were linked with long chain, secondary monols or diols (3,4). Ascarosides in *A. lumbricoides*, a parasite of the pig, resemble those of *P. equorum* (5,6).

Following a suggestion made by Fauré-Fremiet, Fouquey et al. (3) discovered that ascarosides in unsaponified lipid extracts consisted at least in part of acetate and propionate esters. Similar esters from *A. lumbricoides* were said to be esterified in addition to α-methyl butyric and α-methyl valeric acids (6). These branched chain fermentation acids had previously been shown to occur as high molecular weight esters in the neutral lipid fraction which consists chiefly of ascaroside esters and triglycerides (7,8). In ovarian ascarosides, both glycone and aglycone hydroxyl groups may be esterified, whereas ascarosides in the ascaroside

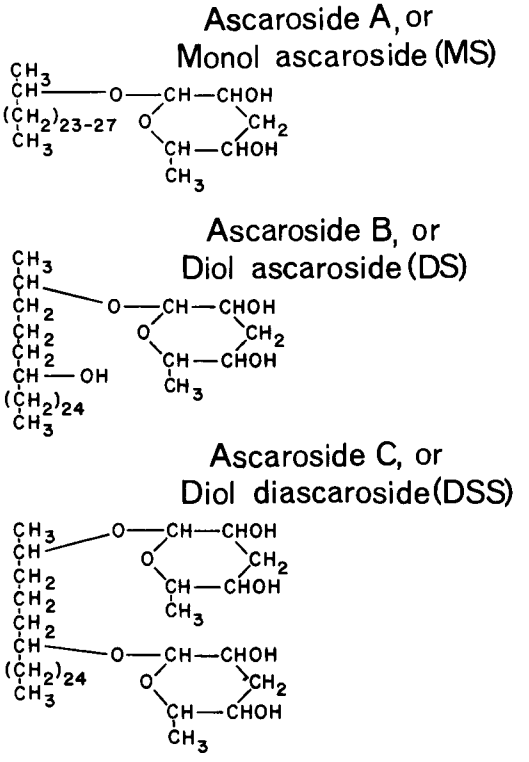

FIG. 1. Ascarosides in the unsaponifiable fraction of *Parascaris equorum* lipids (4).

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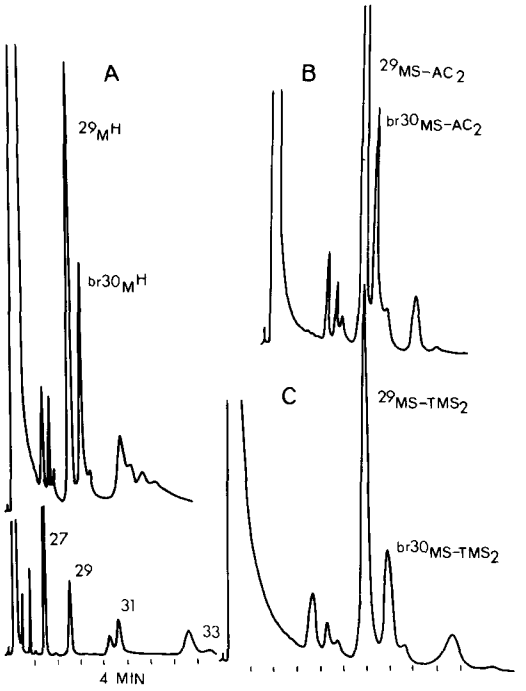

FIG. 2. Gas liquid chromatography on 3% octadecyl silicone of derivatives of the monol ascarosides occurring in *Ascaris lumbricoides* ascarosides. (A) Alkanes derived by reduction of the aglycones, chromatographed at 240 C and N_2 flow rate of 70 ml/min. (B) Naturally occurring monol ascaroside diacetates, chromatographed at 290 C and N_2 flow rate of 66 ml/min. (C) Monol ascaroside trimethylsilyl ethers, chromatographed at 280 C and N_2 flow rate of 70 ml/min. In each chromatogram the three peaks preceding the ^{29}M derivatives are C_{27}, C_{br28}, and C_{28} homologs; the small peak following the ^{br30}M derivative is the C_{30} isomer; and the succeeding two peaks are the C_{31} and C_{br32} homologs, respectively. Two contaminants that may be chlorides are present with the alkanes. In the beeswax hydrocarbons (lower left) that served as standards, the peaks preceding C_{31} and C_{33} are the unsaturated analogs.

layer of the massive egg shell (9) behave chromatographically like the free ascarosides described by Fouquey et al. (3). Some of these nevertheless contain an ester linkage which was thought to consist of long chain fatty acids linked to an aglycone (6).

In the present report we describe details of the composition and structure of the ascaroside esters occurring in *A. lumbricoides*.

MATERIALS AND METHODS

Isolation and Degradation of Ascarosides

Female *Ascaris lumbricoides* were severed at the genital pore, the protruding uterus pulled out with forceps, and the ovary expressed by squeezing the remaining carcass. The ascaroside layer of the egg shell was isolated from the infective eggs (10) that had been shaken with sodium hypochlorite (Clorox) for 24 hr at 4 C in order to dissolve the external, chitinous layer. After thorough washing, the ascaroside layer was ruptured by shaking with aluminum oxide (Alundum, 60 mesh/in.) and separated from the hatched larvae (11).

Total lipids (12) or fractions thereof were saponified with 0.5 N methanolic NaOH for 30 min at 100 C. If the unsaponifiables alone were to be recovered, the digest was extracted with chloroform-water 1:1 v/v, and the chloroform phase containing the unesterified ascaroside was washed free of soaps with 2 N aqueous ammonia. When the saponifiables were also needed for analysis, e.g., in the examination of naturally occurring ascaroside esters, methyl esters formed during saponification were hydrolyzed by heating the digest with 1 volume of water, after which 1 volume of chloroform was added and the volatile acids removed with the upper phase as sodium salts. Both upper and lower phases were then dried at 100 C under nitrogen. Fully esterified ascarosides could be partially saponified by heating the saponification solution for 30 sec at 100 C.

The glycosodic linkages in ascarosides were broken by heating in 1.0 N methanolic HCl for 30 min at 100 C, and partially disrupted by heating for 3 min. Aglycones and partially degraded ascarosides were recovered by adding water and extracting with chloroform. Methyl ascarylose, together with aglycones, was recovered by drying the digestion mixture.

Aglycone hydroxyl groups were reduced by conversion to mesylates and treatment of the mesylates for several hours, at 40 C under nitrogen, with a large excess of a saturated solution of lithium aluminum hydride in tetrahydrofuran (13). The mesylates were formed by treatment with methanesulfonyl chloride-pyridine 1:2 for 10 min at 22 C. Following the reduction, excess reagent was decomposed by addition of ethyl acetate. Several volumes of 0.1 N aqueous HCl were added and the alkanes extracted into hexane. Hydroxyl groups of the glycone were largely regenerated rather than reduced by this procedure (14).

Synthesis of Ascaroside Acetates

As shown by IR spectra, all hydroxyl groups in unesterified ascarosides were converted to acetate esters by heating at 100 C for 10 min with acetic anhydride-pyridine 1:1. Heating for ca. 2 min produced a mixture of partially acetylated ascarosides. Propionate, butyrate, valerate and caproate esters were prepared from the anhydrides by heating for somewhat longer

times. Formate esters were synthesized by heating for 5 min at 100 C with a suspension of 5% sodium methoxide in methyl formate, and mixed esters were synthesized from purified partial esters (see following section) by treatment with selected acid anhydrides. The synthetic esters were extracted into chloroform after diluting the reaction mixture with water.

Thin Layer Chromatography (TLC)

Glass analytical plates (80 x 100 mm) were coated with 0.2 mm Adsorbosil 3 (Applied Science Labs., State College, Pa.). Preparative plates (200 x 200 mm) were coated with 0.5 mm Silica Gel G (Merck).

Hexane, hexane/2-propanol 7:1 and chloroform-methanol 47:3 were used for the fractionation of alkanes, lipids of the ascaroside layer and unesterified ascarosides, respectively. Total lipids, ascaroside esters and aglycones were fractionated by gradient elution TLC (15) in which a volatile, relatively polar trough solvent is gradually replaced on the thin layer plate by a nonpolar solvent saturating the tank. Such a system is represented as atmospheric benzene/ethyl solvent. Although the system most frequently employed was solvent/trough acetate, some separations were achieved with benzene/benzene-butanone 1:2 and hexane/2-propanol. When desirable the gradient range was decreased by adding atmospheric solvent to the trough or vice versa. All plates were developed at ca. 22 C.

Lipids on analytical plates were detected by spraying with sulfuric acid-formaldehyde 97:3 and heating at 200 C. The α-naphthol, anisaldehyde-perchloric acid and diphenylamine sprays for carbohydrates and glycosides were also used (16). Diol aglycones, their volatile acid and sulfonic acid esters, and their glycosides gave blue to violet stains when sprayed lightly with sulfuric acid and exposed for 5-10 min to iodine vapor. The reaction was reversible and was not evident when comparable amounts of other lipids were present in the same area of the plate.

Gas Liquid Chromatography (GLC)

All analyses were carried out in a Barber-Colman Series 5000 chromatograph equipped with a flame ionization detector. Column materials were obtained from Applied Science Labs. Ascarosides and their derivatives were fractionated on a 1.2 m or 1.8 m x 6 mm diameter column packed with 3% octadecyl silicone bonded to Chromosorb W, 80-100 mesh. This packing was prepared according to Aue and Hastings (17), except that the octadecyl trichlorosilane was polymerized for 16 hr in a refluxed and stirred toluene suspension of the support. Free hydroxyl groups were then reacted with dimethyl dichlorosilane, and the chloro groups displaced by 1-butanol. Columns packed with 3% OV-1 on Gas Chrom Z (100-120 mesh) gave similar results, but at higher temperatures and with inferior resolution.

TABLE I

Aglycones Occurring in the Ascarosides of *Ascaris lumbricoides* Ovaries[a]

Carbon no.	Wt % aglycone MS[b]	DS[b]	DSS[b]
25	Trace		
br26	Trace		
26	Trace		
27	4.4		
br28	3.6		
28	1.8		
29	61.8	0.59	Trace
br30	20.5		
30	1.3		
31	6.0	41.9	14.3
br32	0.58		
32	Trace		
33		56.2	81.9
35		1.29	3.8

[a]Aglycones were liberated by acid methanolysis of the unsaponifiables, and reduced to alkanes. Chain lengths were assigned by comparison with the gas liquid chromatography retention times of dotriacontane and purified beeswax hydrocarbons. Branching was inferred from values for equivalent chain length and mass spectrometry. The aglycones were quantified by gas liquid chromatography of their trimethylsilyl ether derivatives. Blank spaces indicate concentrations less than 0.1%

[b]See Nomenclature.

Alkanes and completely esterified ascarosides were chromatographed as such. Alcohols were first converted to trimethylsilyl (TMS) ethers by treatment for 10 min or more at 22 C with pyridine-hexamethyldisilazane-trimethylchlorosilane 9:3:1. This reaction mixture was then injected directly into the column. Column temperatures varied from 240-330 C and nitrogen flow rates from 40-70 ml/min, according to the volatility of the compounds, which was greatest for derivatives of monol aglycone and least for those of diol diascaroside. Dotriacontane (C_{32}) and beeswax hydrocarbons isolated by TLC (18) served as standard alkanes. Peaks were quantified by cutting and weighing. Carbons bonded to two oxygens were considered not to contribute to the response, and the TMS group was counted as two carbons.

Sodium salts of volatile acids, dissolved in 95% formic acid, were injected into a 1.8 m column of 80-100 mesh Chromosorb 101 operated at 180 C and a N_2 flow rate of 60 ml/min. Standard volatile acids were obtained commer-

cially. Peak heights were proportional to amounts except for acetic acid, in which the relative volume of formic acid affected the response and made the use of a correction factor necessary. Formic acid was substituted for aqueous acid because of its superior solvent power for the longer chain acids, and for other solvents because the flame ionization detector was insensitive to it.

Plots of log retention time vs. carbon number were employed as aids in the identification of homologs. Equivalent chain lengths (ECL) for unknowns were read from graphs prepared for known materials.

RESULTS

Nomenclature

Fouquey et al. (3) isolated one monol-containing and two diol-containing ascarosides from the unsaponifiables of *Parascaris equorum* (Fig. 1, ascarosides A, B, and C, respectively). Although the ascaroside layer of the egg shell has been thought to consist primarily of these unesterified ascarosides, its major component is now known to be an ester that in most solvent systems cochromatographs with ascaroside A and has been referred to as such (6). The terms ascaroside A, B and C also make no provision for naming the numerous ascaroside esters to be described below.

In the nomenclature we have employed, M, D and S represent the monol aglycone, diol aglycone and glycone, respectively (Fig. 1). MS, DS and DSS are then the generic descriptors of ascarosides in which the aglycone chain lengths in each group may be variable. A particular chain length may be specified by a prefix, e.g., ^{29}MS, but as this does not appear to be generally useful each group will be referred to in the singular. Esters are designated by adding subscripts and superscripts, representing the acid, to the basic descriptor. For DS the positions by carbon number (3) are $D_6S_4^{2'}$, and diol ascaroside triacetate is $D_{Ac}S_{Ac}^{Ac}$, which may be shortened unambiguously to $D_{Ac}S\text{-}Ac_2$ or $DS\text{-}Ac_3$. A class of isomers is indicated with parentheses: DS-(Ac,Pr). Unless it is stipulated that the first S in DSS stands for the carbon 2 sugar, no distinction will be assumed. Indeed, such distinction might be invalid if, as suggested below, the diol aglycone is actually a symmetrical molecule. Ascaroside derivatives such as mesylates (Ms), trimethylsilyl ethers (TMS) and alkanes (H) are described in the same manner as the esters, as are derivatives of the aglycone or glycone alone, e.g., MeS refers to methyl ascarylose.

It will be shown that all of the ovarian ascaroside esters are absent from the ascaroside layer of the egg and possess at least one ester group on the glycone. Conversely, none of the ascarosides of the fully shelled egg are found in appreciable amounts in the ovaries, and in none is the glycone esterified. To emphasize this distinction and to conform as far as possible with past usage, "ascaroside" will be used as a collective name; "ester" will refer to any derivative with that implied structure; "unesterified ascarosides" will be the products of complete saponification; and a "free ascaroside" will be any ascaroside lacking ester linkages on the glycone. Reference to naturally occurring mixtures may be made in terms of a particular tissue, e.g., "egg ascarosides."

Structure of Unesterified Ascarosides

Thin layer chromatographic analysis of the unsaponifiables obtained from the total lipids of *A. lumbricoides* ovaries showed that three major, nonacidic components were present. These migrated on TLC plates as described previously (6) and stained with all three spray reagents for glycosides. In order of increasing polarity, they were designated MS, DS and DSS.

Gas liquid chromatography of $MS\text{-}(TMS)_2$, naturally occurring $MS\text{-}Ac_2$, and alkanes derived from M gave patterns of peaks that were essentially the same and identifiable without ambiguity with reference to standard alkanes (Fig. 2). Similar identifications were obtained in the analyses of derivatives of DS and DSS. A quantitative summary is set out in Table I. Several minor monol peaks were tentatively identified as branched because their retention times did not correspond with the normal homologs and no unsaturation was found. Equivalent chain lengths for these peaks were ca. 0.4 carbons less than for the presumed normal isomer.

Preliminary evidence from mass spectrometry supported the chain length assignments and suggested that a methyl group occurred on either carbon 2 or 4 of the two major branched monols. In the diols, as in monols, only hydroxyl groups in a carbon 2 position were detected, which would seem to require that the C_{31} and C_{33} homologs be symmetrical 2,30- and 2,32-diols, respectively. Although this would account for the failure of Kirrmann and Wakselman (19) to corroborate, by synthesis and mass spectrometry, Fouquey's placement of the second hydroxyl group on C_6, conclusive information has not yet been obtained.

Acid methanolysis of unesterified ascarosides yielded a pair of glycosidic bands chromatographing on TLC plates above methyl

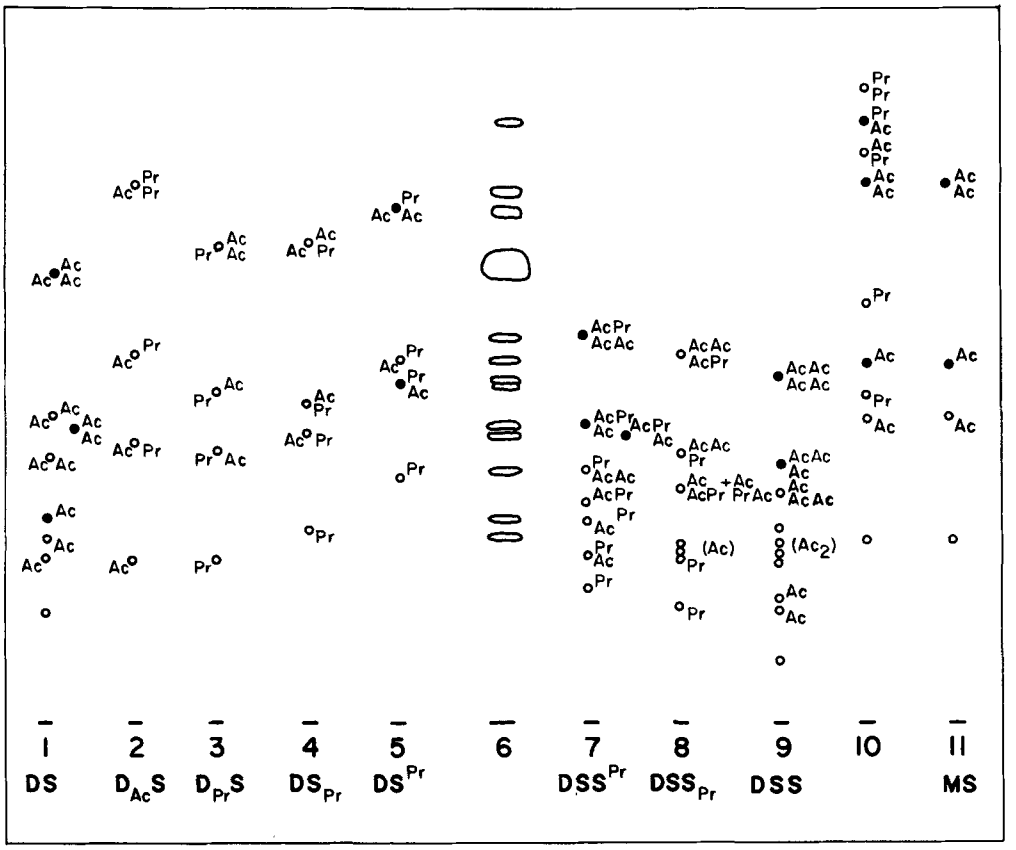

FIG. 3. Thin layer chromatography characteristics of synthetic ascaroside esters and esters isolated from *Ascaris lumbricoides* ovaries. An 80 x 200 mm plate was coated with 0.2 mm Adsorbosil 3 and developed by gradient elution with benzene/ethyl acetate. Synthetic acetates were prepared from the lowermost spot in each column (identified below the column number) except for column 6, which is a reference chromatogram of the ovarian ascarosides of *A. lumbricoides*, and column 10, which was prepared by propionylation of sample 11. Ac appearing on the left of a spot identifies an esterified aglycone; Ac or Pr to the right of a spot identifies a glycone (S) or glycones (SS) esterified in the 2′ position (superscript) or 4′ position (subscript). Naturally occurring esters are designated by •. The lowermost band in column 6 has some properties of ascarosides, but was not otherwise identified. In this figure only the lower two-thirds of the original chromatogram is reproduced. All triglycerides occurred above the uppermost, naturally occurring ascaroside ester. Sterols and diglycerides between DS-Ac_3 and DSS-Ac_4, monoglycerides essentially cochromatographing with $D_{Ac}S$, and free fatty acids at the origin were all minor bands.

L-rhamnose (a monodeoxyhexose) in the region of DS and DSS. TMS derivatives of both glycosides had identical retention times when analyzed by GLC. The bands were presumed to be those of α- and β-methyl ascarylose.

Structure of Synthetic Ascaroside Esters

All synthetic acetates and many mixed acetate-propionates of MS, DS and DSS were prepared, and the positions of the ester groups determined as follows.

Partial acetylation of MS produced two well-spaced bands chromatographing between MS and MS-Ac_2 (Fig. 3, column 11), which clearly demonstrated the separability of the two isomers of glycone monoesters by TLC. Similar treatment of DS (Fig. 3, column 1) produced six bands chromatographing between DS and DS-Ac_3. Therefore the theoretical and observed numbers of partial acetates of DS were also identical. As the number of hydroxyl groups would be expected to be the main determinant of chromatographic mobility, the lower three partial esters should be monoacetates, and the upper three, diacetates. Partial esterification or saponification of purified esters, followed by TLC analysis, supported this supposition and moreover; demonstrated their structural relationships. For example, the highest and lowest diacetates, but not the middle one, were obtained by acetylation of the lowest monoacetate.

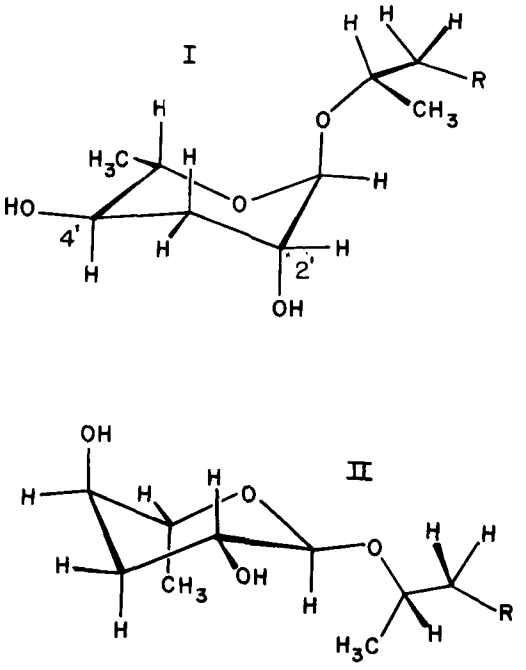

FIG. 4. Chair conformations of ascarylose in L-α-ascarosides. In conformation II, reactions at the 2′ hydroxyl may be sterically hindered by the aglycone.

The partial acetates of DS were sorted into two categories of absolute configuration on the basis of aglycone esterification. Each partial ester was converted to its mesylate and reduced with lithium aluminum hydride. Mesyl groups became -H, while acetate groups became -OH, so aglycones obtained by acid methanolysis were monols if the aglycone was originally unesterified or diols if originally esterified.

The two diacetate mesylates with acetylated aglycones were further employed to distinguish the two glycone esters. Rupture of glycosidic bonds is generally inhibited by a sulfonyl group on C2 of the glycone, although sometimes by this group at other positions as well (14). Sulfonyl groups themselves were found to be resistant to acid methanolysis, so that there are two modes of degradation for the isomers $D_{Ac}S^{Ms}_{Ac}$ and $D_{Ac}S^{Ac}_{Ms}$. Removal of acetate produces monohydroxy, then dihydroxy-ascarosides, whereas rupture of the glycosidic linkage produces monoacetylated, then unesterified, aglycones. All of these could be separated by TLC. Because of its 2′ mesyl group, $D_{Ac}S^{Ms}_{Ac}$ would be expected to show more acetate removal than glycone removal relative to $D_{Ac}S^{Ac}_{Ms}$. This property was possessed by the mesylate of the lower chromatographing $D_{Ac}S$-(Ac), which is therefore $D_{Ac}S_{Ac}$. Comparison of relative rates was necessary, as a continuum of inhibition in these experiments was evident. A mesyl group in either position inhibited rupture of the glycosidic linkage more than did acetate, which was itself inhibitory. Complete configurations are given in Figure 3.

Saponification of DS-Ac_3 confirmed these structures. Partial saponification produced significant quantities only of esters in which the aglycone remained esterified. This was presumably because the reaction of glycone esters was accelerated by the electron-withdrawing groups in adjacent carbon atoms (20). The concentration of the higher chromatographing sugar ester in each pair of isomers was considerably less than that of the lower isomer. As partial esterification gave the same, rather than opposite, relative concentrations, it is clear that the rates of hydrolysis and esterification are not controlled by the same factors. An explanation may be found in the L-α-configuration of ascarosides (4) which permits two chair conformations for ascarylose (Fig. 4). In each conformation there are two equatorial and two axial substituents, and no distinct preference for either would be expected. Steric effects should be of major importance in esterification, while both polar and steric effects should affect saponification (20). In conformation I the aglycone is oriented away from both hydroxyl groups and steric effects should follow the axial-equatorial difference. Esterification of the 4′ carbon should therefore predominate. Conformation II should also favor esterification of the 4′ carbon, as reaction at the 2′ carbon is now hindered by the aglycone. In contrast, polarity is greater at carbon 2′ in both conformations, and 2′ esters should be preferentially saponified.

If the aglycones of DSS are symmetrical (see Nomenclature) there are eight partial acetate configurations; if asymmetrical, there are 14 such configurations. Eight bands appeared on TLC plates, suggesting either that the aglycones are symmetrical, or that the mobility of monoesters of DSS is independent of the particular glycone that is esterified. It was expected that a pair of ester bands nearly cochromatographing with DS would be a mixture of 4 monoacetates. Partial acid methanolysis of the lower, more abundant band produced mainly DS_{Ac} and smaller amounts of DSS and D. The upper band produced DS^{Ac}. The lower band was therefore a mixture of DSS_{Ac} and $DS_{Ac}S$, if these are indeed different isomers. In any case, the results indicated that if the aglycones of DSS are asymmetrical, 6 of the 14 theoretical esters would not be expected to appear separately on the chromatogram and that the observed and expected number of esters was identical.

TABLE II

Ascaroside Esters Occurring in *Ascaris lumbricoides* Ovaries[a]

Structure	Wt % total ascaroside ester	Mol % parent ascaroside
Esters of monol ascaroside		
MS^{Pr}_{Ac}	1.37	14.8
$MS\text{-}Ac_2$	7.64	84.9
MS^{Ac}	0.03[b]	0.3
Total	(9.0)	(10.5)[c]
Esters of diol ascaroside		
$D_{Ac}S^{Pr}_{Ac}$	8.18	10.1
$DS\text{-}Ac_3$	68.0	84.8
DS^{Pr}_{Ac}	0.60	0.54
$DS\text{-}Ac_2$	3.26	4.4
DS^{Ac}	0.074[b]	0.10
Total	(80.1)	(80.9)[c]
Esters of diol diascaroside		
$DS^{Ac_S Pr}_{Ac_S Ac}$	0.92	8.6
$DSS\text{-}Ac_4$	3.04	28.8
$DS^{Ac_S Pr}_{Ac_S Pr} + DS^{Ac_S Pr}_{Ac}$	2.06	17.4
$DS^{Ac_S Ac}_{Ac_S Ac}$	4.58	45.2
Total	(10.6)	(8.6)[c]
Unknown ascaroside	0.26[b]	

[a]Ovarian lipids were fractionated by gradient elution thin layer chromatography with benzene/ethyl acetate. Esters were identified (Fig. 3) and except where indicated, quantities were calculated on the basis of analysis for volatile acids, and average molecular weights. M, monol aglycone; D, diol aglycone; S, glycone; Ac, acetate; Pr, propionate. The subscript after D refers to an esterified aglycone and other subscripts and superscripts refer to glycone positions 4′ and 2′, respectively.

[b]Calculated from gas liquid chromatography analysis of the saponified ascaroside.

[c]Mol % total ascaroside.

By analogy with other pairs of esters, the upper band of the triacetate pair is $DS^{Ac_S Ac}_{Ac_S Ac}$ (no sugar distinction) and the lower is $DS^{Ac_S Ac}_{Ac_S Ac}$. It was not immediately apparent which of the four diacetate bands corresponded to the theoretical esters $DS^{Ac_S Ac}$, $DS_{Ac_S Ac}$, $DS^{Ac_S Ac}$, and DSS^{Ac}_{Ac}. Again, partial acid methanolysis resolved the problem. The highest and least abundant diacetate lost acetate to give only DSS^{Ac}, while the most abundant and second most polar diacetate gave only DSS_{Ac}. Each of the other diacetates yielded both DSS_{Ac} and DSS^{Ac}, but the lower (and lowest of the four) also gave a considerable quantity of $DS\text{-}Ac_2$. The four acetate diesters (Fig. 3, column 9, DSS-[Ac_2]) are therefore, top to bottom, $DS^{Ac_S Ac}$, $DS_{Ac_S Ac}$, $DS_{Ac_S Ac}$, and $DS^{Ac}_{Ac}S$. The low TLC mobilities of $DS^{Ac}_{Ac}S$ and $D_{Ac}S$ suggests an "anomalously" great adsorption of the unesterified glycone to the silica gel. This strength was maintained in alcoholic solvents, which reversed the mobilities of isomeric glycone esters.

These analyses of ascaroside acetates by TLC were sufficiently consistent to permit the assignment of configuration to other esters by analogy. The mobilities and structures of certain mixed acetate-propionate esters that are relevant to the analysis of naturally occurring ascaroside esters are included in Figure 3.

Structure of Naturally Occurring Ascaroside Esters

The neutral lipids of *A. lumbricoides* ovaries gave about nine bands that stained positively with glycoside sprays, and most of these also reacted positively to the diol specific sulfuric acid-iodine reaction. Two major triglyceride bands did not react, nor did several bands occurring at concentrations below those needed for detection by specific reagents. Bands corresponding to free ascarosides were barely detectable.

TABLE III

Aglycones in the Ascaroside Esters of *Ascaris lumbricoides* Ovaries and in the Ascaroside Layer of the Egg Shell

Aglycone chain length[a]	Wt % total aglycone												
	Monol ascarosides		Diol ascarosides				Diol diascarosides				Ascaroside layer ascarosides[b]		
	MS^{Pr}_{Ac}	$MS\text{-}Ac_2$	$D_{Ac}S^{Pr}_{Ac}$	$DS\text{-}Ac_3$	DS^{Pr}_{Ac}	$DS\text{-}Ac_2$	$DS^{AcS^{Pr}_{Ac}}_{Ac}$	$DSS\text{-}Ac_4$	$DSS(Ac_2Pr)$	DS^{AcSAc}_{Ac}	$D_{Ac}S$	DS
27	7.0	5.4										
br28	3.2	3.5										
28	1.8	1.7										
29	60	60	0.6	0.7		0.1					1.1	0.8
br30	24	21										
30	1.5	1.4										
31	3.7	7.1	43	42	24	23	20	19	11	11	35	32
br32	Trace	0.5										
33			56	56	72	72	77	78	86	85	62	62
35			0.7	1.6	4	4.9	3	3	4	4	2.2	4.8

[a]Determined by gas liquid chromatography of trimethylsilyl ether derivatives of the unesterified ascarosides that were obtained by saponification of the esters. Esters within each class are arranged according to thin layer chromatography mobility.

[b]The aglycones of MS and DSS are the same as those shown in Table I.

In determining the structures of the ovarian ascaroside esters, preparative TLC plates were developed with benzene/ethyl acetate. Appropriate bands were re-examined by analytical TLC and if impure, were rechromatographed. Adequate fractionation of a mixture occasionally required an alternate solvent system such as benzene/benzene-butanone 1:2. The pure fractions were then saponified and the acidic and alcoholic components analyzed. The kind and quantity of the volatile acid(s) recovered, the parent ascaroside (only one or none was recovered from each fraction), and comparison by means of TLC of the natural ester with synthetic standards determined a unique formula for each of 15 naturally occurring ascaroside esters (Fig. 3). A specific search for $D_{Ac}S^{Ac}$, whose presence might have been obscured by $DS\text{-}Ac_2$, was unsuccessful. Molecular weights were calculated and used in conjunction with moles of volatile acid to calculate the relative weights of esters in the ovaries (Table II). One ester was not identified. It stained with the glycoside sprays and when saponified yielded acetic acid and a band chromatographing between DS and DSS.

Quantification by volatile acid analysis was confirmed by GLC of the TMS derivatives of intact or saponified esters, although some differences were noted in relative quantities, particularly for esters of DSS. Volatile acid analysis was in general the more reliable method. The presence of a propionyl group in $D_{Ac}S^{Pr}_{Ac}$ and the absence of hydroxyl groups in this and the two other major esters was confirmed by IR spectrometry.

Although 12.3 mol % of the ovarian ascaroside esters contained propionate, none of the esters contained more than one mole of this acid. As a result, acetate accounted for 95.6 mol % of the total ester groups. Because the 5 and 6 carbon acids that are abundant in ovarian triglycerides and waxes (Tarr and Fairbairn, unpublished results) were previously reported to be constituents of ascaroside esters (6) a number of esters of the form $D_{Ac}S^{x}_{Ac}$ (where x equals butyrate, valerate and caproate) were synthesized. These were homologous with the naturally occurring propionyl esters. TLC bands corresponding to such esters were not present in ovarian esters, and the conflict with previous observations is undoubtedly due to the improved techniques devised for use in the present investigation.

The aglycone composition of the 10 most common ovarian esters (Table III) shows that the enzyme(s) synthesizing these esters are sensitive to aglycone chain length. For example, diesters of DS contain 23 wt % of the C_{31}

aglycone, and triesters, 43%. Similarly, triesters of DSS contain 11% of the C_{31} aglycone, and tetraesters, 20%. The conversion of ovarian diesters of DS to DS of the ascaroside layer, and of triesters of DS mostly to $D_{Ac}S$, is reflected in aglycone compositions.

Ascarosides of the Ascaroside Layer of the Egg Shell

Although neutral lipids of the ascaroside layer chromatographed in chloroform-methanol 95:5 gave three bands corresponding to MS, DS and DSS, the MS was judged to be impure, as saponification left a residue of MS and produced a large increment in DS (6). The ascaroside layer therefore contains an ester cochromatographing with MS. When the two compounds were fractionated by use of solvent systems containing an alkane and a ketone, alcohol or carboxylic acid, the ester component cochromatographed with $D_{Ac}S$, $D_{Fo}S$ and was barely separable from $D_{Pr}S$. GLC analysis of the TMS derivatives of all four esters identified the unknown ester as a monoacetate of DS. Upon saponification this ester yielded 0.90-1.06 mol acetate per mole DS, and less than 0.5 mol % of higher acids. Its IR ester absorption band at 8.05 μ corresponded exactly to that of synthetic acetate esters. Formate and propionate esters absorbed at distinctly longer wavelengths. The identification of the ascaroside layer ester as $D_{Ac}S$ was therefore unequivocal.

Relative quantities of the four ascaroside layer ascarosides were determined by gravimetry, GLC of TLC fractions, and by GLC of the TMS derivatives of the total lipids of the ascaroside layer. Each method gave similar results. Weight percentages were MS, 12; $D_{Ac}S$, 70; DS, 10; DSS, 8. Significant amounts of other lipids were not observed in the ascaroside layer, although several minor bands were present on TLC plates. Three of these corresponded to acidic degradation products of ascarosides and may have been artifacts formed during the embryonation of the eggs in 0.1 N sulfuric acid solution. Ascarosides in the total lipids of fully shelled, one-celled eggs taken from the lower part of the uterus were the same as those of the ascaroside layer from infective eggs, and only traces of ovarian-type esters were found. Therefore fertilization and shell formation are associated with profound changes in the ascaroside esters of the ovaries.

DISCUSSION

The present work establishes the chain lengths and relative quantities of the odd-numbered aglycones occurring in the three major ascarosides of *A. lumbricoides*. Modest amounts of branched chain, even-numbered aglycones were tentatively identified (Table I). In the ovaries, where the ascarosides occur almost exclusively as esters, 15 esters were identified in terms of their acetyl and propionyl residues (Fig. 3, Table II). This number would be considerably larger if variability in the aglycone residues was included (Table III). Ovarian triglycerides and waxes are now known to be esterified with the α-methyl butyric and α-methyl valeric acids that were previously thought, owing to incomplete resolution on TLC plates, to occur also in the ascaroside esters. Although the results presented here were based on analyses of pooled lipids from several animals, analyses of individuals has indicated that ester and aglycone compositions are quite constant.

Three fully substituted and 22 partially substituted ascaroside esters of any given acid are possible. Among the esters identified, ca. 91.5 mol % were fully substituted, and 80% of these contained only acetyl residues, a propionyl residue having replaced an acetyl residue in the remainder (Table II). Partially esterified ascarosides with only one free hydroxyl group comprised nearly all the remaining esters. Propionyl groups accounted for only 4.4 mol % of the total ester linkages, and always occurred at the 2′ position of the aglycone.

Esters of the DS class of ascarosides accounted for 80% of all the ovarian ascaroside esters. Most of these contained an acetyl group on the aglycone, which was more resistant to saponification than the other ester linkages (6). As the amount of ovarian ester having unesterified aglycone is sufficient to account for 40% of the ascaroside layer DS, only ca. 9% of the more resistant acetate is hydrolyzed during shell formation. A comparison of the aglycone composition of the ovaries and ascaroside layer ascarosides (Tables I, III) leads to the conclusion that the aglycones are not significantly modified during ascaroside layer formation. From other results (Tarr and Fairbairn, unpublished) it is known that the only function of the ovarian esters is to contribute to egg shell formation. Some 75% of the ascaroside layer consists of ascarosides, and it is this layer that provides an extraordinarily effective barrier between the embryo and the external environment (21).

Previous work supported the conclusion that the ovaries synthesized ascarylose from glucose, and aglycones by a Claisen-like condensation of higher fatty acids, the glycosidic linkage then being formed in the usual manner (22). As it is also known that the in vitro incorporation of

labeled acetate into ascaroside esters is extensive (23), the ovaries contain complete facilities for their synthesis. Condensation of 2 mol of even-numbered fatty acids, with elimination of 1 mol carbon dioxide, satisfactorily accounts for the predominantly odd numbered aglycones (C_{29},C_{31},C_{33}), but does not explain the presence of minor amounts of branched chain, even-numbered aglycones unless, for example, these are formed by condensation of an even numbered acid with a branched chain, odd-numbered acid. Only one branched acid (br15:0) has been found in the ovaries (8,24). The possibility that the aglycone carbon chain may also be synthesized by chain lengthening of acids must be considered, since there is evidence for the presence of a malonyl-CoA pathway in ovaries, and ^{14}C from acetate has been found in GLC eluates corresponding to C_{28} and higher acids (23). Branches could be produced from methyl malonyl CoA, which may be present in significant concentrations (25). Whatever the synthetic scheme, the absence of branching in diols may reasonably be ascribed to interference by the methyl branch with the introduction of a second hydroxyl group.

The presence of acetyl and propionyl groups only in ascaroside esters, and α-methylbutyryl and α-methylvaleryl groups only in the triglycerides and waxes of the same organ, suggests either that highly specific kinases are present or that the steric configuration of ascarosides excludes all but C_2 and C_3 acids. The former possibility seems to be the more likely. In any event, the highly developed preference for the acetyl group may be biologically significant, as egg shell formation requires large amounts of acetate for the synthesis of chitin, and chitin synthesis occurs at the time that the ascaroside esters are being hydrolyzed (presumably by a hydrolase) and the ascaroside layer formed (9,21). Both the chitinous layer and the underlying ascaroside layer are synthesized from yolk materials that are secreted almost immediately after the egg is fertilized, in a closely coordinated sequence of events.

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REFERENCES

1. Flury, F., Arch. Exp. Path. Pharmak. 67:275 (1912).
2. Fauré-Fremiet, E., Arch. Anat. Microsc. 15:435 (1913).
3. Fouquey, C., J. Polonsky and E. Lederer, Bull. Soc. Chim. Biol. 39:101 (1957).
4. Fouquey, C., J. Polonsky and E. Lederer, Ibid. 44:69 (1962).
5. Fouquey, C., Thesis, University of Paris, France, 1961.
6. Jezyk, P.F., and D. Fairbairn, Comp. Biochem. Physiol. 23:691 (1967).
7. Fairbairn, D., Can. J. Biochem. Physiol. 33:31 (1955).
8. Beames, C.G., Exp. Parasit. 16:291 (1965).
9. Foor, W.E., J. Parasit. 53:1245 (1967).
10. Kaulenas, M.S., and D. Fairbairn, Exp. Cell Res. 52:233 (1968).
11. Fairbairn, D., and B.I. Passey, Can. J. Biochem. Physiol. 33:130 (1955).
12. Folch, J., M. Lees and G.H. Sloane-Stanley, J. Biol. Chem. 226:497 (1957).
13. Gaylord, N.G., "Reduction with Complex Metal Hydrides," Interscience Publishers, Inc., New York, 1956.
14. Ball, D.H., and F.W. Parrish, Adv. Carbohydrate Chem. Biochem. 23:233 (1968).
15. Tarr, G.E., J. Chromatogr. 52:357 (1970).
16. Kirchner, J.G., "Thin Layer Chromatography," Interscience Publishers, Inc., New York, 1967.
17. Aue, W.A., and C.R. Hastings, J. Chromatogr. 42:319 (1969).
18. Tulloch, A.P., Lipids 5:247 (1970).
19. Kirrmann, A., and C. Wakselman, Bull. Soc. Chim. Fr. 1967:937.
20. Gould, E.S., "Mechanism and Structure in Organic Chemistry," Holt, Rinehart and Winston, Inc., New York, 1959.
21. Fairbairn, D., Exp. Parasit. 6:491 (1957).
22. Jezyk, P.F., and D. Fairbairn, Comp. Biochem. Physiol. 23:707 (1967).
23. Beames, C.G., B.G. Harris and F.A. Hopper, Ibid. 20:509 (1967).
24. Jezyk, P.F., Can. J. Biochem. 46:1167 (1968).
25. Saz, H.J., and A. Weil, J. Biol. Chem. 237:2053 (1962).

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Effects of Acyl Chain Hydroxyl Groups and Increasing Unsaturation on the Autoxidation of Cholesteryl Esters in Aqueous Colloidal Suspension[1]

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ABSTRACT

Aeration in aqueous collodial suspension results in oxidation of the sterol moiety of cholesteryl linoleate, linolenate, arachidonate and O-tetrahydropyranylricinoleate. The extent of such oxidation is different for each compound, and the O-tetrahydropyranylricinoleate ester oxidizes relatively slowly. Cholesteryl 12-hydroxystearate and ricinoleate are resistant to this oxidation. Factors that may contribute to autoxidative susceptibility of cholesteryl esters are discussed briefly.

INTRODUCTION

In a previous study (1) in this laboratory it was found that esterification of cholesterol with linoleate did not inhibit the susceptibility of the cholesteryl residue to autoxidative attack in an aqueous colloidal suspension, which is in contrast to the lack of autoxidative susceptibility of the more saturated fatty acyl cholesteryl esters. Since autoxidation can occur concurrently in the acyl chain and in the sterol ring system of cholesteryl esters, we shall refer in this report to autoxidation at these two molecular sites as "acyl chain oxidation" and "sterol oxidation." In order to obtain additional information, which might prove helpful in elucidating the mechanism of the sterol oxidation of cholesteryl linoleate under the prescribed conditions, we undertook to study autoxidation of several types of cholesteryl esters.

Since acyl chain oxidation of cholesteryl linoleate would result in introduction of oxygen-functional groups into the acyl chains, with changes in the polarity of the ester molecule and possibly conformational or orientation changes in the colloidal system, we studied two hydroxy-fatty acyl cholesteryl esters. With one of these compounds, a tetrahydropyranyl (OH protected) derivative was also prepared and studied. Cholesteryl linolenate and cholesteryl arachidonate were studied to observe the effect of introduction of additional double bonds in the acyl chain. It would be of interest to study cholesteryl esters of fatty acids with *cis-trans* unsaturation. As yet we have not studied such compounds. Our findings concerning the susceptibility to sterol oxidation of the several additional esters we have studied are presented in this report.

MATERIALS AND METHODS

Cholesteryl Esters

Cholesteryl linoleate: 99% pure, Applied Science Labs., State College, Pa.; mp 41 C uncorr., lit. value, 42 C (2).

Cholesteryl linolenate: Analabs, Inc., North Haven, Conn. 06473, mp 34-35 C, uncorr., lit. value, 32-33 C (2).

Cholesteryl arachidonate: 90% pure, Analabs Inc.

Cholesteryl 12-hydroxystearate: This ester was synthesized by the interesterification procedure of Mahadevan and Lundberg (2) and purified by silicic acid column chromatography. Thin layer chormatography (TLC) of the product showed a single spot, free of the starting materials and cholesterol; the IR spectrum of the compound was identical to that of cholesteryl esters of saturated fatty acids (2) but in addition showed a free hydroxyl absorption band at 3610 cm^{-1}.

Cholesteryl O-tetrahydropyranyl (THP) ricinoleate: Methyl O-THP ricinoleate was prepared by reaction of methyl ricinoleate with dihydropyran in the presence of concentrated HCl. The product, dissolved in petroleum ether, was washed with saturated sodium bicarbonate solution and water and dried over anhydrous Na_2SO_4. Evaporation of the solvent under reduced pressure yielded methyl O-THP ricinoleate. This was interesterified (2) with cholesteryl acetate. The reaction product, extracted with petroleum ether, was a syrupy residue. This was purified by separation as a noncrystalline phase from 95% ethanol at 10 C (four times). Removal of solvent, drying under

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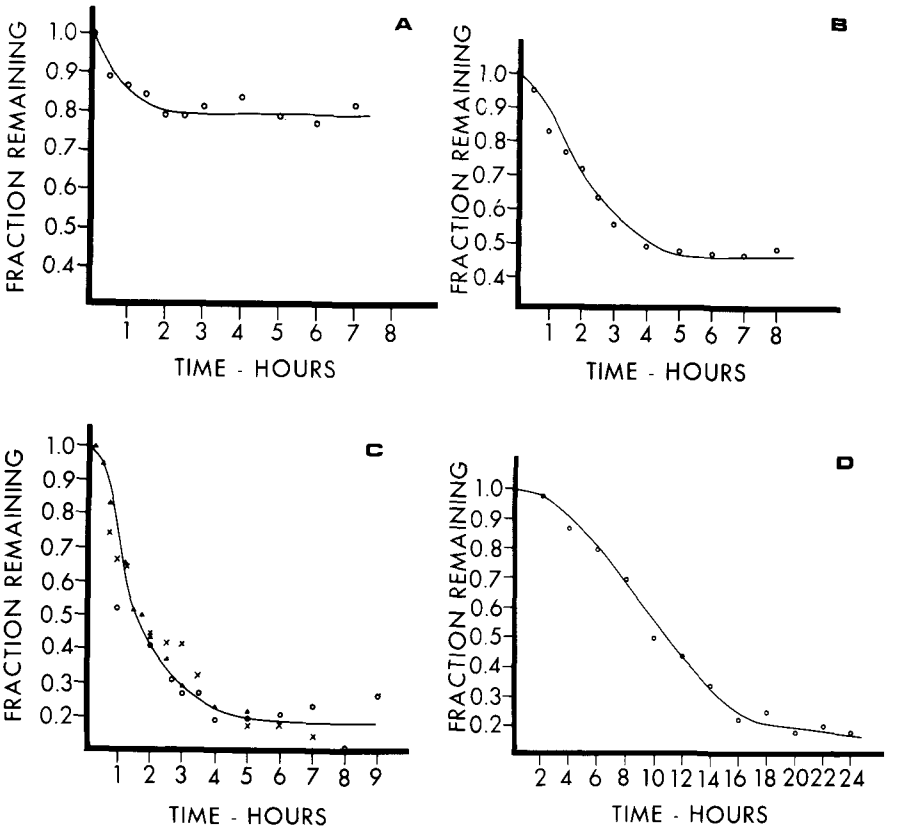

FIG. 1. Fraction of cholesterol remaining (1.0-0.0) during aeration of cholesteryl esters in aqueous colloidal suspension at 85 C; A, cholesteryl arachidonate; B, cholesteryl linolenate; C, cholesteryl linoleate (values from three separate experiments are plotted; and D, cholesteryl O-tetrahydropyranylricinoleate.

vacuum, followed by chromatography on a column of Florisil yielded the final product, cholesteryl O-THP ricinoleate, which was shown to be free of cholesteryl acetate, O-THP ricinoleate and cholesterol by TLC.

Cholesteryl ricinoleate: Cholesteryl ricinoleate was prepared by removal of the THP group from cholesteryl O-THP ricinoleate by acid hydrolysis. The residue obtained following this reaction was dissolved in petroleum ether and washed with a saturated solution of sodium bicarbonate and then with water and dried over anhydrous $MgSO_4$. The petroleum ether solution was passed through a column of silicic acid. By appropriate elution of the column and removal of solvent from the eluate, pure cholesteryl ricinoleate was obtained. TLC of the compound showed a single spot free of the unhydrolyzed compound. $[\alpha]^D$ = -21.0° (C, 5% in $CHCl_3$).

At the time of the autoxidation experiments, the esters used were again studied for the absence of free cholesterol by digitonin precipitation and by TLC.

Autoxidation Conditions

The procedure of autoxidation was similar to that of Bergström and Wintersteiner (3). The contents of the reaction vessel at the outset of each experiment included: 1000 ml distilled H_2O, 30 ml of 1% (w/v) sodium dodecyl sulfate in repurified absolute ethanol and 10 ml of M/15 phosphate buffer of pH 7.0. The pH of the combined flask contents was then adjusted to pH 7.0 ± 0.2 with the addition of dilute HCl or NaOH as needed.

In most of the experiments, 100-400 mg cholesteryl ester in 60 ml repurified absolute ethanol was added to the reaction flask. In a few of the early studies, up to 120 ml ethanol was used to introduce the ester. The mole ratio of sodium dodecyl sulfate to cholesteryl ester in the reaction flask varied from ca. 1.7-6.8, depending on the type and amount of cholesteryl ester used in the particular experiment. It may be calculated from data published by Hartley (4) that the dodecyl sulfate concentration in the reaction flask did not exceed one-eighth the critical micelle concentration at

85 C.

The reaction flask was maintained at 85 ± 2 C in a water bath, and the flask contents were constantly stirred. After introduction of the ester with stirring and removal of the zero time aliquots, aeration of the suspension through a fritted glass gas-washing tube was begun. The air used was bubbled through successive towers of 35% KOH, concentrated H_2SO_4, and distilled water before passing into the reaction vessel. Then 25 ml aliquots of flask contents were removed from the reaction flask at appropriate time intervals for chemical analysis for unoxidized sterol. Four drops concentrated HCl and 10 ml acetone were added to the aliquot in a glass-stoppered flask to stop the reaction, and the flasks were then placed in a refrigerator at 4 C until extracted and analyzed.

Analysis for Sterol Oxidation

The aliquots from the autoxidation reaction vessel were extracted and analyzed as described previously (1,5). More recently Horvath (6) has compared the chemical assay method we are using with a TLC method of studying the extent of autoxidation of cholesterol. In studies having widely varying amounts of autoxidized cholesterol, Horvath's results show reasonably good agreement between the TLC method and the procedure we are using.

RESULTS

Data for the autoxidation of the cholesteryl esters that were susceptible to sterol oxidation are presented in Figure 1. The time courses of sterol oxidation of the linoleate (Fig. 1C), linolenate (Fig. 1B) and arachidonate (Fig. 1A) esters are similar, in that autoxidation occurs in a period of several hours and then the reaction stops. The extent of sterol oxidation for each of the above three esters was different. Conversion to autoxidation products was 81% for linoleate, 54% for linolenate and 21% for arachidonate. Data from three separate experiments are plotted for linoleate (Fig. 1C), primarily to obtain data beyond 5 hr.

The O-tetrahydropyranyl cholesteryl ricinoleate (Fig. 1D) underwent extensive sterol autoxidation but at a much slower rate. Sterol oxidation began early and proceeded at a regular rate for 16-18 hr with a subsequent leveling off of the reaction. The extent of conversion to sterol autoxidation products was in excess of 80%.

Two of the cholesteryl esters studied were completely resistant to sterol oxidation during an 8 hr period of aeration under the specified conditions. These esters were cholesteryl ricinoleate and cholesteryl 12-hydroxystearate.

DISCUSSION

The differences observed in the extent of sterol oxidation of the several polyunsaturated fatty acyl esters studied are of some interest, but they have no obvious explanation. Two factors possibly related to these differences are: (a) extent and time course of free-radical formation in the acyl chain with resultant intramolecular propagation of sterol oxidation, and (b) extent and time course of introduction of oxygen-functional groups in the acyl chain with resultant changes in conformation or orientation of the ester molecules, such that sterol oxidation is influenced. With regard to factor (a), free radicals in acyl chains may both form and then terminate more quickly in the more highly unsaturated fatty acyl cholesteryl esters. This would account for the diminishing extent of sterol oxidation observed for cholesteryl linolenate and arachidonate, compared to linoleate. With regard to factor (b), oxidative introduction of an oxygen functional group into the acyl chain might inhibit subsequent sterol oxidation. This might also explain inhibition of sterol oxidation of arachidonate and linolenate compared with linoleate. Oxygen functional groups would be expected to be introduced more quickly into arachidonate and linolenate acyl chains than into linoleate acyl chains. Our observation of the resistance to sterol oxidation of the ricinoleate and 12-hydroxystearate esters may be taken as one piece of evidence that autoxidative introduction of an oxygen-functional group into the acyl chain of cholesteryl linoleate, for example, does not by virtue of a polarity change cause enhancement, and may cause inhibition, of susceptibility of the molecule to sterol oxidation.

Another hypothesis relating to the susceptibility to sterol oxidation of diethenoyl and polyethenoyl esters, as opposed to monoethenoyl and saturated acyl esters, has to do with the curved conformation of acyl chains having several methylene-interrupted *cis* double bonds. The bending of these chains might serve to prevent packing of the steryl esters and thus allow sterol oxidation. It would be necessary here to invoke involvement of another factor, such as inhibition of oxidation by introduction of an oxygen functional group into the acyl chain, to account for the diminished extent of sterol oxidation of say the arachidonate ester (more acyl chain bending) vs. the linoleate ester (less acyl chain bending). Our results with O-tetrahydropyranyl ricinoleate ester may be

interpreted as favoring this hypothesis, since it seems possible that the introduction of the bulky, hydrophobic, tetrahydropyranyl group onto the acyl chain may alter the conformation or orientation of the cholesteryl ester, such that packing is prevented and the ester becomes susceptible to sterol oxidation. In the case of the O-THP ester, the absence of introduction of oxygen functional groups into the acyl chain by autoxidation may then permit extensive sterol oxidation, as we observed, without inhibition secondarily.

Yet another experimental condition needs to be considered in assessing pertinent factors. During aeration of the colloidal suspension, a bubble or foam layer ca. 1 in. thick persists at the top of the liquid phase. In the foam layer the cholesteryl ester is probably present in a film, as opposed to being in a colloidal particle in the liquid phase. Probably the ester molecules are in exchange between the foam and liquid phases, and it is not known at which site most of the oxidation occurs.

At this stage of our studies it appears that several factors, as discussed above, may be operative in the autoxidative phenomena observed. Studies with additional esters and employing other approaches may provide answers.

The findings we have reported represent an extension of our knowledge concerning autoxidation susceptibility of cholesteryl esters to sterol oxidation in aqueous suspensions. However the reason(s) why some of these esters have a unique susceptibility is still unknown. It seems possible that more knowledge about the factor(s) contributing to this susceptibility may be useful information which may be applied to the study of cholesteryl esters in lipid aggregates, e.g., at biomembranes or reactions of steryl esters with membrane-bound enzymes.

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REFERENCES

1. Norcia, L.N., and W.F. Janusz, JAOCS 42:847 (1965).
2. Mahadevan, V., and W.O. Lundberg, J. Lipid Res. 3:106 (1962).
3. Bergström, S., and O. Wintersteiner, J. Biol. Chem. 145:327 (1942).
4. Hartley, G.S., "Aqueous Solutions of Paraffin-Chain Salts," Hermann & Cie., Paris, 1936, p. 35.
5. Norcia, L.N., JAOCS 30:238 (1961).
6. Horvath, C., J. Chromatogr. 22:52 (1966).

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7-Methyl-7-Hexadecenoic Acid: Isolation from Lipids of the Ocean Sunfish *Mola mola* (Linnaeus) 1758

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ABSTRACT

A fatty acid amounting to several per cent of the acids in various samples of the liver oil of the ocean sunfish *Mola mola* was isolated and characterized as 7-methyl-7-hexadecenoic acid. This acid and the previously reported 7-methyl-6-hexadecenoic acid are believed to be chiefly responsible for the 7-methylhexadecanoic acid observed in many fatty acid mixtures of marine origin after hydrogenation.

INTRODUCTION

The ocean sunfish *Mola mola* is a morphological curiosity. Among major pelagic fish species it is also unusual, in that it is reputed to feed on jellyfish (1). It therefore seemed logical to extend to this species our interest in marine animals originating or accumulating *trans*-6-hexadecenoic acid (2-6). Fortunately the ocean sunfish is usually obtainable in Nova Scotian waters in the late summer or early fall, and a suitable specimen for lipid study was procured. Unexpectedly it was found that the anticipated *trans*-6-hexadecenoic acid was accompanied by another unusual component fatty acid, possibly present in trace amounts in other marine lipids, which has now been identified as 7-methyl-7-hexadecenoic acid.

EXPERIMENTAL PROCEDURES

The lipid investigated came from an ocean sunfish (no. Hx-2) caught thirty miles southwest of Halifax, N.S., on July 30, 1971. The specimen weighed 90 kg, was 125 cm in length, 75 cm in depth and 20 cm in thickness. It was a female, but the gonads were not ripe. The liver (4.5 kg) yielded 55.6% lipid, a clear yellow oil, when extracted by the method of Bligh and Dyer (7). The Wijs iodine value of this oil was 161, and the percentage of unsaponifiable material 5.3% by AOCS Method Ca-6b-53. Fatty acids recovered after removal of the unsaponifiable material were esterified for analytical gas liquid chromatography (GLC) by refluxing for 10 min with 7% BF_3 in methanol. For preparative scale studies esters were prepared by refluxing the acids for 3 hr with methanol containing 1% H_2SO_4.

Sunfish liver oil fatty acid methyl ester mixture (2.47 g) was heated with a saturated solution of urea in methanol (70 ml). This was cooled to room temperature for 1 hr and filtered. Addition of water to the filtrate and extraction with petroleum ether yielded 0.74 g non urea-complexing esters. These were fractionated by preparative thin layer chromatography (TLC) on plates impregnated with silver

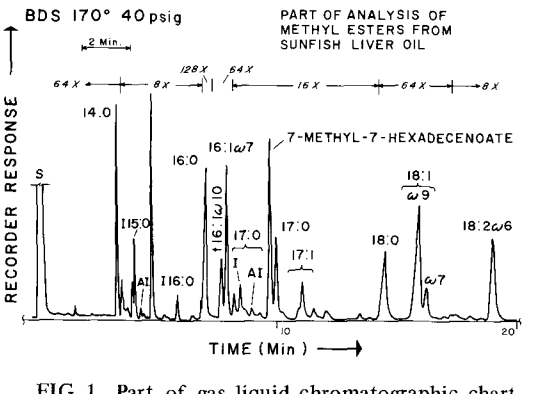


FIG. 1. Part of gas liquid chromatographic chart from the analysis of methyl esters of fatty acids from the liver oil of the ocean sunfish *Mola mola* on a BDS-coated open-tubular gas liquid chromatographic column.

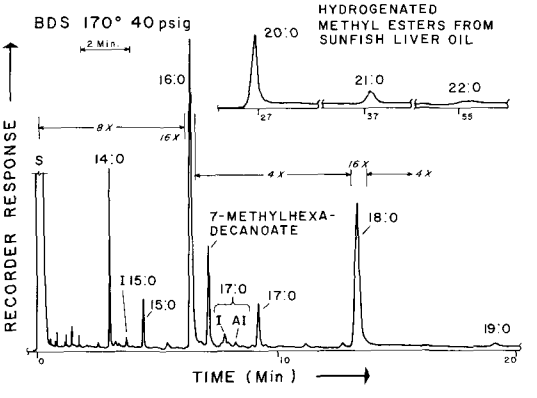


FIG. 2. Gas liquid chromatographic chart of the analysis of the hydrogenated methyl esters of fatty acids from the liver oil of the ocean sunfish *Mola mola* on a BDS-coated open-tubular gas liquid chromatographic column.

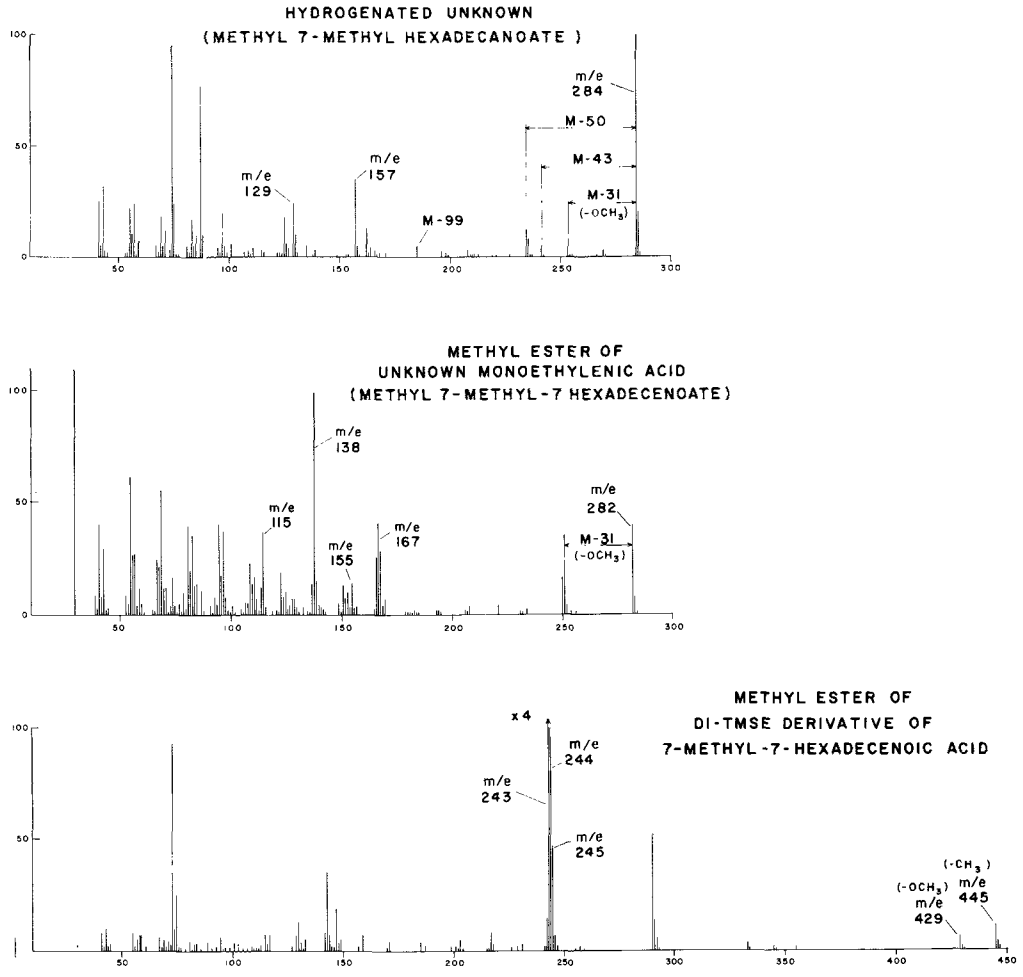


FIG. 3. Mass spectra of derivatives of 7-methyl-7-hexadecenoic acid. Top: methyl 7-methylhexadecanoate. Center: methyl ester of 7-methyl-7-hexadecenoic acid. Bottom: di-trimethylsilylether of methyl 7,8-dihydroxy-7-methylhexadecanoate.

nitrate. After development in *n*-hexane-benzene 1:1 the band at R_f 0.6 was scraped off and extracted three times with hexane-diethyl ether. It contained a major unknown component with equivalent chain length (ECL) (8) of 16.88 on butanediol succinate (BDS) and 16.40 on Apiezon-L (AP-L) with a purity of >95% as determined by open-tubular GLC. Part of the total fatty acid methyl esters from sunfish liver oil was hydrogenated, treated with urea-methanol to remove straight chain components, and the major residual component with ECL of 16.30 (on BDS) or 16.33 (on AP-L) isolated by preparative GLC.

Analytical GLC was carried out on stainless steel open-tubular columns 150 ft (ca. 50 m) in length x 0.01 in. (ca. 0.25 mm) ID coated with BDS, diethyleneglycol succinate (DEGS) or AP-L. These were operated in a Perkin-Elmer Model 900 with the injection port at 270 C. Operating conditions were, respectively, helium carrier gas at 40 and 60 psig (AP-L); and respective column temperatures of 170 and 180 C (AP-L).

Preparative GLC was performed using a W.G. Pye Series 105, Model 15, instrument fitted with a glass column 2 m in length x 1 cm OD, packed with 3% OV-17 on 100/200 mesh Gas-chrom Q (Applied Science Lab., Inc.). This was operated isothermally at 180 C. Other conditions were: injection port 300 C, detector oven 200 C, nitrogen carrier gas 200 ml/min and a 25:1 split between the collector and detector.

TLC was used to examine the liver oil and also to fractionate methyl esters of fatty acids

for identification purposes. Prekote plates (Adsorbosil-5, Applied Science Labs.) were developed in petroleum ether-diethyl ether-acetic acid 85:15:1. Plates coated with silicic acid impregnated with silver nitrate were prepared from Supelcosil 12D (Supelco, Inc.) and developed in *n*-hexane-benzene 1:1.

Ozonolysis of isolated unknown (ECL on BDS 16.88) was carried out with oxidative work-up (9). The products were examined on a glass column 6 ft in length x 0.3 mm ID packed with 5% SE-30 on Gas-Chrom Q, operated in a Varian Hi-Fy at 150 C. Authentic standards for comparison included di- and monocarboxylic acids, normal hydrocarbons (for Kovats Index determinations) and methyl ketones such as 2-nonanone, etc.

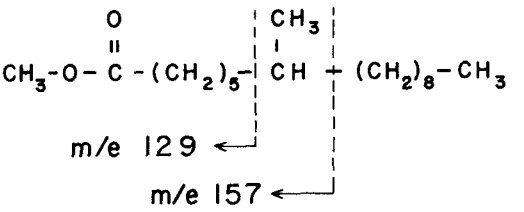
The unknown fatty acid methyl ester was converted to the dihydroxy derivative and thence to the di-trimethylsilylether (di-TMSE) derivative by the method of Capella and Zorzut (10). Mass spectra of the unknown, the dihydro form and the di-TMSE derivative were recorded on a DuPont CEC21-110B double focusing mass spectrometer at 70 eV. Samples were inserted into the source at 30 C using a variable temperature probe.

The NMR spectra of the unknown fatty acid methyl ester and reference materials were run on a Varian A-60 spectrometer in deuterated chloroform.

IR spectra of the unknown fatty acid, as the methyl ester, and of squalene were run on a Unicam SP 100 as a film on a salt block.

RESULTS AND DISCUSSION

The liver oil was shown by TLC to be virtually pure triglyceride with a fatty acid composition similar to most marine oil triglycerides (8,11). Qualitatively, two unusual features were apparent in the 16:0-17:0 region of the chromatogram on BDS (Fig. 1). These were, respectively, the anticipated peak ahead of the 16:1ω7 peak (shorthand for chain length: number of double bonds and position of double bond closest to the terminal methyl group [12]), which could be expected if *trans*-6-hexadecenoic acid were present, and another peak ahead of the 17:0, with ECL of 16.88, in a position commonly occupied by minor and trace components usually tentatively identified as 16:2ω4, 16:2ω6, etc., depending on the polarity of the column. On hydrogenation this component disappeared and a peak of equivalent size was found at ECL 16.30 in the hydrogenated product (Fig. 2). On Apiezon-L open-tubular columns the ECL values were,



Scheme 1.

respectively, 16.40 for unhydrogenated material (after isolation) and 16.30 for hydrogenated material (a single peak in the total esters or also after isolation).

Initially it was thought that the unknown component at ECL 16.88 would be the methyl ester of 7-methyl-6-hexadecenoic acid isolated by Sano from the blubber oil of an arctic finwhale *(Balaenoptera physalus)* and also shown to occur in blubber oil of the sperm whale *(Physeter catodon)* (13). The GLC properties agreed with this view, and in the initial step in the identification, TLC on $AgNO_3$/silica gel, the unknown had the same R_f value as *trans*-6-hexadecenoic acid, this corresponding approximately to the R_f position found by Sano. The ECL for the unknown after hydrogenation, 16.30 on both BDS and AP-L columns, indicated a methyl branch in the C_5, C_6, C_7, C_8 or possibly the C_9 position, but eliminated all other positions for a monomethyl branch, and also ruled out most possible isoprenoid structures (8,14,15).

The NMR spectrum showed the presence of only one olefinic proton (δ5.10, triplet, J = 6 Hz), thus indicating that the methyl branch was located on the double bond. Moreover, the 3 proton signal at δ1.57 is consistent with a methyl group allylic to a double bond.

The IR spectrum showed absorption bands typical of a fatty acid methyl ester with absorption at 840 cm^{-1} due to C-H out of plane deformation of a trisubstituted ethylenic double bond, a weak absorption at 1670 cm^{-1} due to C=C stretching and a doublet at 1380 cm^{-1}, characteristic of a methyl branching. These three bands were also found in the spectra of squalene run as reference standard.

The hydrogenated compound isolated (ECL on BDS of 16.30) (Scheme 1) gave a molecular ion at m/e 284 and typical methyl ester details (Fig. 3). There were intense fragment ions at m/e 157 and 129. These ions are consistent with cleavage α to a branched methyl group as described by Ryhage and Stenhagen (16).

The mass spectra of the original unsaturated unknown (ECL on BDS of 16.88) gave a molecular ion at m/e 282 tentatively suggesting

the C_{17} mono-olefinic methyl branched structure found by Sano. Moreover the spectrum (Fig. 3) strongly resembled that published for methyl 7-methyl-6-hexadecenoate in many details, giving intense peaks at m/e 138, 167, 115 and 155, as also reported by Sano (13). Due to the lability of the olefinic bond upon electron impact (17,18), the location of the unsaturation could not be determined directly from the unsaturated acid methyl ester, and an isomeric structure would likely give many corresponding spectral features. The mass spectra for methyl 3,7,11-trimethyldodeca-6,10-dienoate, and an isomeric form with the central double bond in a vinylic side chain (19) both show many features in common, and these are also similar to the lower molecular weight fragments of the original monoethylenic unknown (Fig. 3). This is a further indication of the difficulty of assigning the location of ethylenic unsaturation by mass spectra.

The di-trimethylsilylether of the dihydroxy derivative (Fig. 3) typically exhibited strong M-15 (m/e 445) and M-31 (m/e 429) peaks. The most intense ions appeared at m/e 243-245 corresponding to cleavage between the two trimethylsilyloxy groups (16). This result unexpectedly showed the double bond to be in the C_7-C_8 position and not in the anticipated C_6-C_7 position (compare [13]).

Ozonolysis confirmed that the structure had a 7-methyl substituent and unsaturation in the C_7-C_8 position. Of the two major oxidation products, nonanoic acid was identified as the methyl ester by direct comparison of retention times with methyl nonanoate. The other major product had a Kovats Index (20) of 13.10 on an AP-L coated open-tubular column. The value calculated for methyl 7-keto-octanoate from retention data for methyl esters of normal acids and of 2-ketones was 13.14. If adipic acid or 2-undecanone (from the isomeric acid isolated by Sano) were included in the products the proportions were minor (<5%), although their absence could not be positively demonstrated.

Most of the fatty acids in samples of marine lipids examined in our laboratory are converted to methyl esters and hydrogenated. Many such samples have been noted to contain small proportions of a peak corresponding to methyl 7-methylhexadecanoate (11,21-25), although this was in the past always thought to arise from the methyl 7-methyl-6-hexadecenoate reported by Sano. The latter acid has been tentatively identified in this laboratory in a sperm whale oil sample in a proportion similar to that of the 7-methyl-7-hexadecenoate isomer. The origins and reasons for specific accumulations in some samples of either or both the 7-methyl-6-hexadecenoic and 7-methyl-7-hexadecenoic acids remain obscure, although the latter may be in some way linked to the coelenterates, specifically jellyfish, suspected to form a part of the diet of the ocean sunfish (1). This view is supported by the definite presence of the *trans*-6-hexadecenoic acid in the sunfish liver oil, as this acid has now been confirmed as a component fatty acid in jellyfish (4).

REFERENCES

1. MacGinitie, C.E., and N. MacGinitie, "Natural History of Marine Animals," Second Edition, McGraw-Hill, New York, 1968, p. 435.
2. Hooper, S.N., and R.G. Ackman, Lipids 5:288 (1970).
3. Hooper, S.N., and R.G. Ackman, Ibid. 6:341 (1971).
4. Hooper, S.N., and R.G. Ackman, Ibid. 7:624 (1972).
5. Ackman, R.G., S.N. Hooper and W. Frair, Comp. Biochem. Physiol. 40B:931 (1971).
6. Ackman, R.G., and S.N. Hooper, Int. J. Biochem. 3:171 (1972).
7. Bligh, E.G., and W.J. Dyer, Can. J. Biochem. Physiol. 37:911 (1959).
8. Ackman, R.G., in "Progress in the Chemistry of Fats and Other Lipids," Vol. 12, Edited by R.T. Holman, Pergamon Press, 1972, p. 165.
9. Ackman, R.G., and J.D. Castell, Lipids 1:341 (1966).
10. Capella, P., and C.M. Zorzut, Anal. Chem. 40:1458 (1968).
11. Ackman, R.G., and C.A. Eaton, Can. Inst. Food Technol. J. 4:169 (1971).
12. Holman, R.T., in "Progress in the Chemistry of Fats and Other Lipids," Vol. 9, Part 1, Edited by R.T. Holman, Pergamon Press, 1966, p. 3.
13. Sano, Y., Yukagaku 16:605 (1967).
14. Ackman, R.G., J. Chromatogr. 42:170 (1969).
15. Ackman, R.G., J. Chromatogr. Sci. 10:243 (1972).
16. Ryhage, R., and E. Stenhagen, Arkiv Kemi 15:291 (1960).
17. Holman, R.T., and J.J. Rahm, in "Progress in the Chemistry of Fats and Other Lipids," Vol. 9, Part 1, Edited by R.T. Holman, Pergamon Press, 1966, p. 15.
18. Zorzut, C.M., and P. Capella, Riv. Ital. Sost. Grasse 46:66 (1969).
19. Ahlquist, L., and S. Ställberg-Stenhagen, Acta Chem. Scand. 25:1685 (1971).
20. "The Practice of Gas Chromatography," Edited by L.S. Ettre and A. Zlatkis, Interscience Publishers, New York, 1967, p. 381.
21. Ackman, R.G., J.C. Sipos and C.S. Tocher, J. Fish. Res. Bd. Canada 24:635 (1967).
22. Sipos, J.C., and R.G. Ackman, Ibid. 25:1561 (1968).
23. Ackman, R.G., J.C. Sipos and P.M. Jangaard, Lipids 2:251 (1967).
24. Ackman, R.G., and S.N. Hooper, Comp. Biochem. Physiol. 32:117 (1970).
25. Ackman, R.G., and S.N. Hooper, Ibid. 24:549 (1968).

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Sphingolipids of the Fungi *Phycomycetes blakesleeanus* and *Fusarium lini*

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ABSTRACT

Ceramides and cerebrosides isolated from *Phycomyces blakesleeanus* and *Fusarium lini* together comprise ca. 0.2-0.3% of the cell dry weight, which is ca. 7-8% of the wet weight. The long chain bases obtained from these sphingolipids are (A) 16-methyl-4-hydroxy heptadecasphinganine; (B) 4-hydroxy octadecasphinganine; (C) 17-methyl-4-hydroxy octadecasphinganine; (D) 18-methyl-4-hydroxy nonadecasphinganine; (E) 19-methyl-4-hydroxy eicosasphinganine; (F) 20-methyl-4-hydroxy heneicosasphinganine; (G) 20-methyl-4-hydroxy heneicosa-X_1-sphingenine; (H) 20-methyl-4-hydroxy-heneicosa-X_2-sphingenine; (I) 4-hydroxy docosasphinganine; (J) 4-hydroxy docosa-X_1-sphingenine; and (K) 4-hydroxy docosa-X_2-sphingenine (The position, geometry and nature of unsaturation are unknown. The subscripts indicate the presence of two different unsaturated bases.) The occurrence in biological materials other than mushrooms of bases (D), (F), (G), (H), (I), (J) and (K) has not been previously reported. (The nomenclature used is that recommended by IUPAC-IUB Commission on Biochemical Nomenclature. The configurations at carbon atoms 2, 3 and 4 are unknown in all cases.) The major normal fatty acids are palmitate, stearate, octadecenoate and octadecadienoate in *P. blakesleeanus* and stearate in *F. lini*. The predominant hydroxy fatty acids are hydroxypentadecanoate and hydroxypalmitate in *P. blakesleeanus* and hydroxy stearate in *F. lini*. The hexose from the cerebrosides of both fungi is glucose, except cerebroside II of *P. blakesleeanus* which contains glucose and galactose.

INTRODUCTION

The isolation and partial characterization of the sphingolipids from cultivated edible and wild mushrooms was reported previously (1). Since mushrooms occupy the highest class, Basidiomycetes, of the order Eumycophyta (true fungi), it was decided to investigate for comparative and classification purposes the sphingolipids of the lowest, most primitive class of true fungi, Phycomycetes, and fungi of uncertain classification, Fungi imperfecti.

TABLE I

Isolation Procedure and Yields of Sphingolipids from *Phycomyces blakesleeanus* and *Fusarium lini*

Procedure	*Phycomyces blakesleeanus*, g	*Fusarium lini*, g
1. Cell wet wt[a]	671.0	1550.0
2. Cell dry wt	47.2	124.0
3. Acetone soluble material	12.3	13.7
4. Chloroform-methanol 2:1 soluble material	7.7	10.9
5. Lipids after Folch dialysis of 3. and 4. combined	12.8	14.3
6. Silicic acid chromatography		
Neutral lipids	5.9	10.8
Polar lipids	3.2	2.5
Polar lipids after NaOH	2.0	1.7
7. Silicic acid chromatography of polar lipids		
Ceramides	0.170	0.170
Cerebrosides	0.080	0.150
8. Florisil chromatography		
Ceramide I	0.0736	0.0945
Ceramide II	0.0221	0.0751
Cerebroside I	0.0220	0.0314
Cerebroside II	0.0413	0.0385

[a]See text for details.

TABLE II

Long Chain Bases and Alcohols Derived from Sphingolipid Fractions of *Phycomyces blakesleeanus*

Fraction	Phytosphingosine[a,b]	Per cent	Long chain alcohol[a,c]	Per cent
Ceramide	*i*17:0	3.7	*i*14:0	1.2
	*n*18:0	21.8	*n*15:0	22.8
	*i*18:0	14.0	*i*15:0	14.8
	*i*19:0	8.0	*i*16:0	11.7
	*i*20:0	8.5	*i*17:0	15.6
	*i*21:0	39.0	*i*18:X_1	2.4
			*i*18:X_2	21.8
			*i*18:0	4.5
	*n*22:0	5.0	*n*19:X_1	2.0
			*n*19:X_2	3.2
Cerebroside	*i*17:0	3.1	*i*14:0	4.0
	*n*18:0	31.7	*n*15:0	9.2
	*i*18:0		*i*15:0	1.2
	*i*19:0	19.6	*i*16:0	25.4
	*i*20:0	32.5	*i*17:0	32.6
	*i*21:0	7.3	*i*18:X_1	3.7
			*i*18:X_2	9.2
			*i*18:0	3.6
	*n*22:0	5.8	*n*19:X_1	2.8
			*n*19:X_2	6.0
			*n*19:0	2.3

[a]Ceramides I and II and cerebrosides I and II obtained from Florisil chromatography, Table I, were analyzed for long chain bases, derived long chain alcohols and fatty acids. The values obtained for each component from each of the sphingolipid pairs were averaged and presented in the appropriate tables.

[b]Bases were obtained from sphingolipids hydrogenated over platinum by acid hydrolysis and analyzed by gas chromatography as trimethylsilyl derivatives.

[c]Intact sphingolipids were degraded with periodic acid, and the isolated aldehydes were reduced with sodium borohydride to alcohols which were determined by gas chromatography as trimethylsilyl derivatives. The subscripts represent different alcohols of unknown unsaturation. See text for details.

Phycomyces blakesleeanus and *Fusarium lini* were chosen as representatives of the former and latter classes, respectively.

METHODS AND MATERIALS

Column (CC), thin layer (TLC) and gas (GC) chromatographies and IR spectroscopy were performed as described previously (1). Column fractions were analyzed for long chain bases (LCBs), serine and ethanolamine (H.L. Meltzer, unpublished results), hexose (2), phosphate (3) and ester groups (4). Ascending TLC with solvent systems chloroform-methanol-H_2O 100:42:6 (5) and chloroform-methanol-28% NH_4OH 65:35:5 (6) was used to monitor the purity of fractions obtained by CC, and preparative TLC with the former solvent was employed when further purification was necessary. Components were detected by a variety of reagents (7) but preferably by iodine vapor. A 6.0 ft, 0.25 in. ID glass column packed with 3% SE-30 on Gas Chrom Q was used for the GC determination of LCBs, long chain alcohols and simple sugars as their trimethylsilyl (TMS) derivatives (8-10); fatty acids were determined as methyl or ethyl esters and hydroxy fatty acids as methyl esters-TMS ethers. Column temperatures of 195 C and 240 C were employed for determining bases of chain length up to C-18 and beyond C-18, respectively. The lower and upper ranges of the series of normal and iso alcohols of chain length C-14 to C-21 were analyzed at 150 C and 165 C, respectively. Fatty acids and sugars were analyzed at column temperatures of 165 C and programing from 100-210 C at 2 C/min, respectively.

Reference compounds were obtained as follows: Cerebrosides were prepared from bovine brain (11); sphingosine was isolated as the sulfate salt after acid hydrolysis of cerebrosides (11); dihydrosphingosine was obtained by reduction of sphingosine free base in ethanol over platinum; O-methyl ethers were isolated as described earlier (12,13); phytosphingosine was obtained from the yeast *Hansenula ciferri* (14); the ceramides N-myristoylsphingosine and N-palmitoyldihydrosphingosine were synthesized (15); psychosine, fatty acid esters and α- and β-methyl glycosides were either commercial

TABLE III

Long Chain Bases and Alcohols Derived from Sphingolipid Fractions of *Fusarium lini*

Fraction	Phytosphingosine	Per cent	Long chain alcohol	Per cent
Ceramide	*i*17:0	15.5	*i*114:0	19.6
	*n*18:0	63.4	*n*15:0	43.8
	*i*18:0	1.6	*i*15:0	3.3
	*i*19:0	9.1	*i*16:0	14.8
	*i*20:0	8.8	*i*17:0	14.8
	*i*21:0	1.6	*i*18:X_1	2.3
			*i*18:X_2	0.3
			*i*18:0	0.3
			*n*19:X_1	0.3
			*n*19:X_2	0.5
Cerebroside	*i*17:0	10.5	*i*14:0	10.6
	*n*18:0	20.9	*n*15:0	10.9
	*i*18:0	16.3	*i*15:0	10.6
	*i*19:0	2.8	*i*16:0	10.9
	*i*20:0	38.4	*i*17:0	30.2
	*i*21:0	11.1	*i*18:X_1	2.3
			*i*18:X_2	19.4
			*i*18:0	5.1

products or prepared; normal alcohols were purchased, and branched chain iso and anteiso alcohols were prepared from their corresponding branched chain fatty acid mixtures BC-1 and BC-L from Applied Science Labs (1,5).

The logarithms of the retention times of standard LCBs vs. their carbon numbers were plotted to obtain equivalent chain lengths (ECL). This plot was used to calculate the ECLs of unknown LCBs. Due to the lack of several LCB standards, the retention data presented by Carter and Gaver (9) was adapted for our use.

Isolation of Sphingolipids

Nine 2.5 liter Erlenmeyer flasks, each containing 1 liter of modified Czapek-Dox medium (16), were inoculated with a spore-mycelial suspension of NRRL 2204 *F. lini* and shaken at constant temperature of 28 C for 2 weeks. The mycelia were removed by filtration on a Buchner and washed on the funnel with several liters of distilled water. *Phycomyces blakesleeanus*, ATCC 6200, was grown, also, on a defined medium (17,18), and the mycelia were treated in the same manner as that used for *F. lini*. After homogenization of each washed fungus in 500 ml acetone in a Waring blender and filtration, the cells were treated two additional times with the same volumes of acetone and three times with chloroform-methanol (C/M) 2:1. The combined acetone and C/M filtrates were concentrated separately to dryness under reduced pressure. To the combined acetone and C/M residues from each fungus were added 160 ml chloroform, 80 ml methanol and 60 ml water for a Folch dialysis (19); the upper phase was discarded, and the same amount of fresh upper phase was added. After mixing and centrifugation, the lower phase was concentrated to dryness. The residue was loaded from chloroform on a 60 g silicic acid column, the neutral lipids were eluted with 1500 ml chloroform and the polar lipids with 1 liter methanol.

After removal of methanol, the polar lipids were subjected to mild alkaline hydrolysis followed by a Folch dialysis (1,5). The residue from the lower phase was reapplied from chloroform to a 30 g silicic acid column. After passage of 1 liter chloroform (discarded), the column was developed successively with 750 ml each of C/M 96:4 and C/M 80:20 to remove ceramides and cerebrosides, respectively. The ceramide and cerebroside fractions, after removal of solvent, were loaded on separate 10 g Florisil columns (20,21). The ceramide-containing column was treated successively with 100 ml chloroform (discarded) and 175 ml each of C/M 96:4 and C/M 42:4 to give ceramide I and ceramide II fractions, respectively. Similarly, the column containing cerebrosides was developed successively with 100 ml C/M 96:4 (discarded) and 175 ml each of C/M 42:4 and CM/ 2:1 to yield cerebroside I and cerebroside II fractions, respectively.

Hydrolysis of Sphingolipids

Fractions, 2-5 mg, were hydrolyzed in 2 ml concentrated HCl/methanol/water 3:29:4 in a heating block at 78 C for 18 hr, and the fatty acids and LCBs were isolated (1,5) and analyzed as described above. A separate sample of

TABLE IV

Fatty Acids of *Phycomyces blakesleeanus*

Fatty acid	Ceramide, %	Cerebroside, %
*n*12:0	1.4	0.2
*n*13:0	1.4	1.4
*n*14:0	2.5	1.5
*i*14:0	0.5	0.6
*n*15:0	3.2	2.3
HO-15:0	9.8	1.3
*n*16:0	21.0	17.9
*n*16:1	2.9	0.6
HO-16:0	1.0	41.5
*n*18:0	11.8	7.6
*n*18:1	31.2	15.0
*n*18:2	6.8	7.1
*n*18:3	0.5	0.4
HO-18:0	0.7	1.0
*n*19:0	2.9	
*n*19:1	0.7	
*n*20:0	1.1	1.2
*i*20:1	0.6	0.4

TABLE V

Fatty Acids of *Fusarium lini*

Fatty acid	Ceramide, %	Cerebroside, %
*n*12:0		0.2
HO-*n*12:0	0.4	Trace
*n*13:0	0.1	0.2
HO-*n*13:0	0.1	Trace
*n*14:0	0.4	0.2
*i*14:0	0.8	0.1
HO-*n*14:0	Trace	0.1
*n*15:0	0.8	0.2
HO-*n*15:0	0.1	0.4
*n*16:0	2.0	0.2
*i*16:0	0.1	0.1
HO-*n*16:0	12.4	2.3
*n*17:0	0.4	0.1
*n*18:0	6.4	0.4
*n*18:1	1.2	0.4
*n*18:2	1.1	0.5
HO-*n*18:0	71.1	94.2
*n*19:0	0.2	0.3
*n*20:0	2.4	0.1

cerebroside was methanolyzed in anhydrous IN HCl/methanol for sugar analyses (1,10).

Reduction of Sphingolipids

Intact lipids, 3-5 mg in 2 ml ethanol containing 3 mg platinum oxide, were hydrogenated at 35 C for 2 hr according to the procedure described previously (1). The reduced lipids were hydrolyzed in the same manner as that used for the unreduced lipids.

Periodate Oxidation Sodium Borohydride Reduction

Intact unreduced and reduced lipids, 1-3 mg, were suspended in 2 ml methanol/water 9:1 followed by the addition of 0.15 ml of 0.2 N aqueous periodic acid. After warming the reaction mixture at 40 C for 15 min and standing at room temperature for 2 hr, the long chain aldehydes were isolated and reduced to alcohols (1,5).

Partial Fractionation of Neutral Lipids

After removal of chloroform, 3.0 g neutral lipid from *F. lini* were loaded on a 30 g silicic acid column which was successively developed with 500 ml portions of heptane and chloroform. The residue from the heptane eluate contained long chain fatty acid ethyl esters which were analyzed by GC. A portion of these ethyl esters were hydrolyzed with mild alkali at room temperature, and the free fatty acids were reesterified with diazomethane.

RESULTS

Approximately 92-93% of each fungus is water (Table I). The neutral and polar lipids of *P. blakesleeanus* are 12.0 and 6.0% respectively, of the cell dry weight, whereas in *F. lini* the neutral and polar fractions are 9.0 and 2.0%, respectively. The final combined ceramides and cerebrosides from *P. blakesleeanus* and *F. lini* represent ca. 0.4 and 0.2% of the dry weight, respectively. Ceramides comprise ca. 60% in *P. blakesleeanus* and 70% in *F. lini* of the total sphingolipids.

After hydrolysis of the ceramides and cerebrosides from *P. blakesleeanus* and *F. lini,* the bases obtained are all phytosphingosine homologs ranging in length from C-17 to C-22 (Tables II, III). Except for the presence of normal 18:0 and 22:0, all of the bases are of the iso series. In the ceramides and cerebrosides of *P. blakesleeanus,* the major bases are *n*18:0, *i*20:0 and *i*21:0 (Table II). The dominant bases of *F. lini* are *n*18:0 and *i*20:0. The alcohols obtained from periodate oxidation of the intact lipids followed by sodium borohydride reduction of the isolated aldehydes disclosed two unknown unsaturated components each in the *i*18 and *n*22 series (Tables II, III). These bands disappear into their corresponding analogs after similar treatment of oxidation and reduction of the reduced intact lipids. In those cases where all three alcohols are present in either the *i*18 or *n*19 series, the major component is the corresponding $i18/X_2^x$ or $n19/X_2^x$, respectively.

Palmitic, stearic, oleic and linoleic acids are the major normal fatty acids in *P. blakesleeanus* (Table IV); the dominant hydroxy fatty acids are hydroxypentadecanoate in ceramide II and hydroxypalmitate in cerebrosides I and II. The major fatty acid of the sphingolipid fractions of *F. lini* is hydroxystearate.

The sugar present in the cerebrosides of both

fungi is glucose, except for cerebroside II of *P. blakesleeanus,* which contains equal amounts of glucose and galactose.

The free fatty acid ethyl esters from the neutral lipid fraction of *F. lini* are *n*14:0, *n*15:0, *n*16:0, *n*16:1, *n*18:0, *n*18:1 and *n*18:2; their percentages are 3.1, 3.1, 25.5, 2.0, 10.2, 30.6 and 25.5, respectively. These esters were confirmed by the similar retention times of authentic ethyl esters and by comparison with known methyl esters, after conversion of thy unknown ethyl esters to methyl esters before and after hydrogenation.

DISCUSSION

In recent studies of the total lipids of the cultivated mushroom, *Agaricus bisporus* (22) sphingolipids were not found; nor were they reported in the polar lipids, which comprised ca. 40% of the total lipids, of the thermophilic fungus *Humicola grisea* (23). In the current investigation, it is firmly established that sphingolipids, as ceramides and cerebrosides, are present in fungi of the most primitive class, Phycomycetes, as well as in the most complex class, Basidiomycetes (1). Since these lipids represent ca. 0.003% of the wet weight, they may have been easily overlooked if insufficient amounts of material were processed. The overall pattern of base composition, which has no obvious symmetry, in *P. blakesleeanus* and *F. lini* resembles generally, with some base omissions, that of the mushrooms (1).

The divergence in some cases between the percentages of LCBs and their corresponding alcohols is attributed mainly to differential destruction of bases, primarily the unsaturated compounds of the *i*21 and *n*22 series, during hydrolysis of the sphingolipids. As a result of this variable degradation, the LCB data are presented in the tables in their reduced form. Similarly, the alcohols obtained from the free bases, after periodate oxidation and sodium borohydride reduction, do not correspond to the complete pattern of alcohols which is obtained by treatment of the intact lipids with these reagents. The superiority of the Carter and Hirschberg procedure (5) for the identification of LCBs by degradation to identifiable alcohols is due to the mild conditions employed. Had identification of the LCBs been sought via oxidation of the derived long chain aldehydes to their corresponding carboxylic acids by present methods, the unsaturated components of the *i*18 and *n*19 series would probably not have been observed. One possible difficulty in employing the alcohol procedure for routine identification may be in the occurrence of LCBs of low molecular weight; these may yield partially volatile alcohols which would be lost during processing. Thus the most reliable and reproducible data regarding the composition of phytosphingosines in the sphingolipids of fungi are those derived from the alcohols after appropriate treatment of the intact lipid. No dihydroxy bases were found after hydrolysis of either the unreduced or reduced lipids.

The bases previously found in other biological materials (24) and present in *P. blakesleeanus* and *F. lini* are: (A) 16-methyl-4-hydroxy heptadecasphinganine; (B) 4-hydroxy octadecasphinganine; (C) 17-methyl-4-hydroxy octadecasphinganine; and (D) 19-methyl-4-hydroxy eicosasphinganine. The new bases, which have not been previously reported other than in mushrooms, are: (E) 18-methyl-4-hydroxy nonadecasphinganine; (F) 20-methyl-4-hydroxy heneicosasphinganine; (G) 20-methyl-4-hydroxy heneicosa-X_1-sphingenine; (H) 20-methyl-4-hydroxy heneicosa-X_2-sphingenine; (I) 4-hydroxy docosasphinganine; (J) 4-hydroxy docosa-X_1-sphingenine; and (K) 4-hydroxy docosa-X_2-sphingenine.

The general structure strongly indicated for the phytosphingosines in the various sphingolipid fractions is 1,3,4-trihydroxy-2-amino-*n*- or *i*-alkane; the unsaturated members may contain single or multiple olefinic or acetylenic bonds, or both. The arguments for this conclusion are the same as those presented earlier (1). Problems to be completed regarding the bases are determination of: (a) the configuration of carbon atoms 2, 3 and 4; (b) position, geometry and nature of unsaturation in bases of the *i*21 and *n*22 series; and (c) anomeric configuration of the glycosidic linkage in the cerebrosides. Similarly, the position and configuration of the hydroxyl groups and double bonds of the fatty acids also remain to be determined to establish their structures unequivocally.

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REFERENCES

1. Weiss, B., and R.L. Stiller, Biochemistry 11:4552

(1972).
2. Radin, N.S., F.B. Lavin and J.R. Brown, J. Biol. Chem. 217:789 (1955).
3. Marinetti, G.V., J. Lipid Res. 3:1 (1962).
4. Meltzer, H.L., J. Biol. Chem. 233:1327 (1958).
5. Carter, H.E., and C.B. Hirschberg, Biochemistry 7:2296 (1968).
6. Rouser, G., C. Galli and G. Kritchevsky, JAOCS 42:404 (1965).
7. Mangold, H.K., in "Thin Layer Chromatography. A Laboratory Handbook," Edited by E. Stahl, Springer-Verlag, New York, 1969, p. 363.
8. Gaver, R.C., and C.C. Sweeley, JAOCS 42:294 (1965).
9. Carter, H.E., and R.C. Gaver, J. Lipid Res. 8:391 (1967).
10. Dijong, I., P.T. Mora and R.O. Brady, Biochemistry 10:4039 (1971).
11. Carter, H.E., W.P. Norris, F.J. Glick, G.E. Phillips and R. Harris, J. Biol. Chem. 170:269 (1947).
12. Carter, H.E., O. Nalbandov and P.A. Tavormina, Ibid. 192:197 (1951).
13. Weiss, B., Biochemistry 3:1288 (1964).
14. Weiss, B., and R.L. Stiller, Lipids 5:782 (1970).
15. Weiss, B., and P. Raizman, J. Amer. Chem. Soc. 80:4657 (1958).
16. Fiore, J.V., Arch. Biochem. 16:161 (1948).
17. Bernhard, K., L. Abisch and H. W. gner, Helv. Chim. Acta 40:1292 (1957).
18. Garton, G.A., T.W. Goodwin and W. Lipinsky, Biochem. J. 48:154 (1951).
19. Folch, J., I. Ascoli, M. Lees, J.A. Meath and F.N. LeBaron, J. Biol. Chem. 191:833 (1951).
20. Wells, M.A., and J.C. Dittmer, J. Chromatogr. 18:503 (1965).
21. Kishimoto, Y., and N.S. Radin, J. Lipid Res. 1:72 (1959).
22. Holtz, R.B., and L.C. Schisler, Lipids 6:176 (1971).
23. Mumma, R.O., R.D. Sekura and C.L. Fergus, Ibid. 6:589 (1971).
24. Karlsson, K.A., Ibid. 5:878 (1970).

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Addition Compounds of Oxidizing Tocopherol and Soybean Lecithin[1]

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ABSTRACT

Evidence is presented for the formation of addition compounds from tocopherol and soybean lecithin, when adsorbed in monolayer on silica from a mixed solution in chloroform and oxidized at 80 C for 72 hr. Tocopherol was present at 7 mol % of the lecithin in the monolayer. The compounds produced are analogous to linoleic acid-tocopherol adducts previously reported by us. UV spectra of the mixed lecithin and tocopherol monolayers while in silica gel slurry in a solvent were obtained by the method previously reported by us for oxidized linoleic acid and tocopherol monolayers. The monolayer spectra show no evidence for tocopherol dimer. A minor amount of quinone is indicated, but the spectrum has a maximum at 287 nm with no other major peaks. The proposed addition compounds have been characterized by transesterification and hydrogenation, UV, IR, thin layer chromatography and gas chromatography retention behavior, mass spectrometry and elemental analysis. IR spectra of twice-chromatographed addition compounds show specific lecithin absorption bands (5.75 μ; 10.3 μ) together with much enhanced 3.4-3.5, 6.8, 7.25 and 9.14 μ peaks, the latter two being characteristic of α-tocopherol. The UV spectrum of the adducts showed $\lambda_{max}^{CHCl_3}$ 287 nm, with shoulders at 276 and 300 nm. Hydrogenation removed the peaks at 287 and 276 nm, leaving a peak at 300 nm similar to that of the linoleic acid-tocopherol adduct. Esters of the adducts prepared by transesterification and hydrogenation were similar by all our chemical, chromatographic and spectral tests to the previously characterized esters of the linoleic acid-tocopherol adduct.

INTRODUCTION

Skinner and Parkhurst (1) have summarized the main oxidation products of α-tocopherol, other than the well known dimers and trimers. Adducts with other molecules are seldom shown. 5-Benzoyl-oxymethyl-dl-α-tocopherol and the 5-alkoyloxymethyl derivatives have been prepared. A styrene adduct was prepared

[1]Paper No. TP-1225 in the U.S. Army Natick Labs. Series.

TABLE I

Yields of Lecithin-Tocopherol Adducts

Experiment	Yield, mg[a]	Per cent C_1	Per cent C_2
1	2.7	---	---
2	4.0	24	76
3A	3.7	47	53
3B	2.4	---	---
4A	3.1	34	66
4B	3.8	39	61
5	3.4	42	58
Mean ± SD	3.3 ± 0.6		

[a]From a monolayer of 200 mg lecithin and 8 mg tocopherol adsorbed on 1 g silica. See text for source of C_1 and C_2.

TABLE II

Comparative Thin Layer Chromatography on Silica G

System	Concentration	R_f
HEX/EE/HAc	95:5:1	0.31
HEX/EE/HAc	80:20:1	0.59
PE/EE/HAc	30:70:1	0.92

TABLE III

Tocopherol Adsorbed on Silica from Chloroform Solution[a] in the Presence of Lecithin

	Tocopherol adsorbed, mg/g silica	
Experiment	Silica UV	Ethanol extraction and Emmerie-Engel assay
1	13.4	---
2	8.3	7.2
3	8.5	6.7
4	6.8	---
5	7.6	---
Mean	7.8 ± 0.8[b]	7.0 ± 0.3[c]

[a]Adsorbing solution contained for each experiment, 200 mg each of tocopherol and lecithin in 20 ml chloroform with 1 g acid-washed Silica G.

[b]Mean and standard deviation of last four experimental values.

[c]Average error $\pm\Sigma[d]/n$.

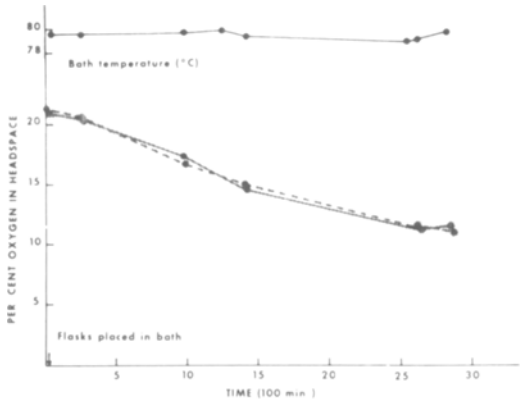

FIG. 1. Oxygen uptake during oxidation of a mixed lecithin and tocopherol monolayer at 80 C. Tocopherol, 7 mol %. Shaded area includes data of three experiments with at least two samples each. Circles show data from one experiment.

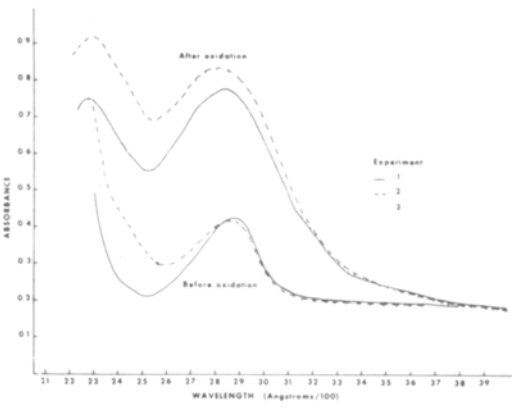

FIG. 2. UV absorption spectra in silica slurry of adsorbed mixed lecithin and tocopherol monolayer. Postoxidation sample withdrawn after 48 hr at 80 C.

by Nilsson et al. (3). Skinner and Parkhurst (4) have prepared adducts with dihydropyran and tetracyanoethylene. Cyclic 5-methyleneoxy-6-oxy-7,8-dimethyl tocol phosphate has been prepared (1). Komoda and Harada (5) have reported an adduct of tocored and soybean oil. Gardner et al. (6) have found an adduct of α-tocopherol and linoleic acid hydroperoxide.

We have reported the formation of an adduct of linoleic acid and tocopherol (2), when these compounds are adsorbed from a mixed solution containing 5 mol % tocopherol in a monolayer on activated silica gel and oxidized in air at 80 C for 72 hr. We report herein evidence for the formation of adducts of oxidizing tocopherol and soybean lecithin similar to the reaction reported for linoleic acid and tocopherol.

EXPERIMENTAL PROCEDURES

Materials

Silica Gel G for monolayer adsorption, column chromatographic silicic acid, Sephadex LH-20 and Silica G for thin layer chromatography (TLC), petroleum ether (PE) and nitrogen were procured, purified and treated as reported (2,7).

Chloroform, ACS reagent grade (Allied Chemical, Morristown, N.J.) was used for the adsorbing solutions and for chromatography. It was redistilled before use. Benzene, ethyl ether (EE, Merck and Co., Inc.) hexane (HEX), and methanol, ACS reagent grade (Allied Chemical) and 2,2-dimethoxypropane (Aldrich Chemical Co.) were used as received. Alumina (Fisher Scientific Co., Alumina Adsorption, for chromatographic analysis, 80-200 mesh) was slurried in chloroform and washed on the column with 3 volumes of chloroform before use. Palladium, 10% on powdered charcoal (Matheson, Coleman and Bell), was used as received.

Soybean lecithin (Alcolec gum) was procured from American Lecithin Co. The lecithin was dissolved as a 5% (w/v) solution in chloroform and purified by three repetitions of chromatography on alumina (8). The resulting material gave a single spot on TLC (Silica G-chloroform-methanol-water 65:25:4). Fatty acid analysis of the purified lecithin was by transesterification with BCl_3/MeOH at reflux temperature for 50 min and gas chromatography (GC) on a diethylene glycol succinate column (HI-EFF-1BP, 15% adsorbed on Gas-Chrom P, Applied Science Labs.). Average mol per cent of the major fatty acids were stearic, 6.2; palmitic, 21.7; oleic, 13.4; linoleic, 53.6; and linolenic, 5.1. Calculated molecular weight based on this analysis: 770. Analysis: Calculated for $C_{43.1}H_{78.2}O_8P_1N_1$: C, 67.2; H, 10.2; P, 4.0. Found: C, 67.05, 66.92; H, 10.75, 10.80; P, 3.97, 3.90.

The d-α-tocopherol was procured and purified as in (2). UV, IR and silica slurry UV spectrophotometry were performed as described before (2,9).

Adsorption and Oxidation Methods

The method of anaerobic adsorption of lipid monolayers was reported in (7). In the present study, however, the adsorbing solution contained 200 mg purified soybean lecithin and 200 mg of d-α-tocopherol in 20 ml redistilled chloroform, contained in a 25 ml flask with ground glass stopper. One gram of quadruply acid-washed Silica Gel G activated at 110 C for

1 hr was used as adsorbent. Amounts of tocopherol adsorbed were determined by ethanol extraction and Emmerie-Engel assay (10), by silica-UV spectrophotometry (9), and confirmed by solution-UV spectrophotometry on the supernatant solutions at adsorption equilibrium. Gravimetric measurements on the latter were used to determine lecithin adsorption by difference.

The oxidation of the lipid monolayers at 80 C and monitoring of oxygen in the headspace by gas partition chromatography was carried out as reported (2).

Column Chromatography

Chromatography of oxidized products was done in two stages (1). In the first stage, silicic acid and the eluants benzene, chloroform and chloroform-methanol were used. Since the oxidized products exist in monolayer on the silica, they may be eluted by benzene directly from the dry coated silica used as a support for oxidation, onto the silicic acid-benzene column for chromatography (2). Dimensions of the silicic acid column bed were 2.5 x 9 cm.

Three relatively nonpolar products derived only from tocopherol are eluted by benzene at 0.75, 1.25 and 2.0 bed volumes, respectively. They are well separated and are, in order of increasing elution volume, compounds X, Y and authentic tocopherol. The IR and UV spectra and behavior in TLC and gel filtration chromatography of compounds X and Y are similar to those of the two tocopherol trimer isomers (11). About 1.4 mg of each of these compounds is obtained from the 200 mg lecithin and 8 mg tocopherol present in the monolayer. Minor amounts of other tocopherol-derived compounds are eluted in later benzene fractions, one having a quinone moiety by TLC, IR and UV spectral evidence, but not identical with α-tocopherol quinone.

Adducts of tocopherol with lecithin appear only after prolonged elution of the silica column with chloroform-methanol mixtures. Typically, about two to three bed volumes of chloroform-methanol 2:1 are required. The adducts appear just ahead of authentic lecithin and are merged with it in later fractions.

The second stage of chromatography was gel filtration of the above adduct fractions on Sephadex LH-20 in chloroform. This cleanly separates the adducts from authentic lecithin. A Chromatronix 1 in. chromatography column with Teflon injection valves and tubing (Chromatronix Inc., Berkeley, Calif.) was used. The bed had dimensions of 2.54 x 44 cm. The relevant collected fractions from the silicic acid column were dissolved in 2 ml chloroform and passed through a sintered glass filter before column injection. The adduct products, of which there seem to be at least two species (C_1 and C_2), appear at total bed volumes of ca. 0.6 and 0.8, compared to 1.0 for lecithin. The two species are not completely separated by the gel. Table I shows the yields of adduct obtained.

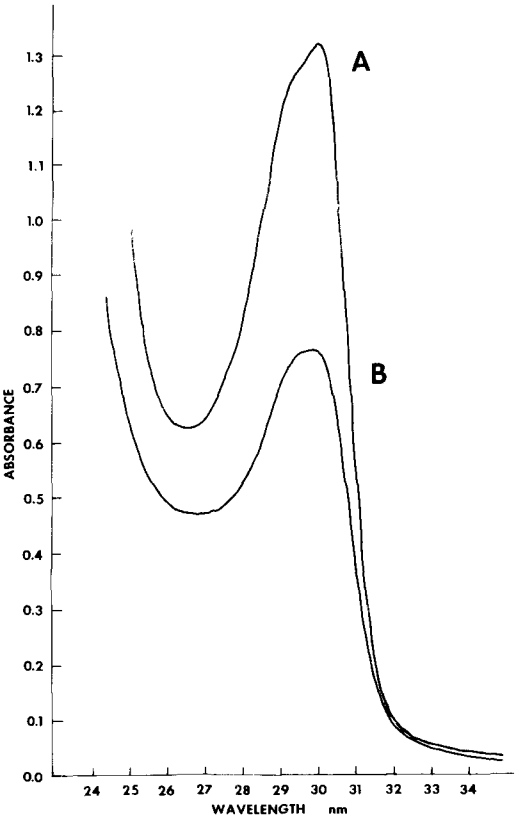

FIG. 3. UV spectra of: (A) ester of linoleic acid-tocopherol adduct; (B) ester of lecithin-tocopherol adduct. Solvent $CHCl_3$.

Characterization of Products

TLC of the adducts on Silica G with chloroform-methanol-water 65:24:4 after successive separation on silicic acid and gel filtration columns showed a single spot for one of the adduct species, C_2, and a somewhat streaked spot for C_1. Mean R_f's of three runs were: C_1 0.46; C_2 0.36; lecithin 0.30. The shape of the spots in two dimensional TLC suggested that C_2 may be a single compound, while C_1 may be a mixture of adduct species; but neither can be regarded as a pure compound, as will be seen below.

The adducts were pale yellow oils with UV $\lambda_{max}^{CHCl_3}$ 287 nm; IR (μ) phosphatide carbonyl, 5.75; methyl bend 7.25 (s); aromatic ether

TABLE IV

IR Absorbance Ratios[a] of Critical Peaks for Lecithin and Adducts

Compounds	$\delta CH_2/\nu CO$ 6.84μ	SD[b]	$\nu CH_2/\nu CO$ 3.45μ	SD	$\delta CH_3/\nu CO$ 7.25μ	SD	10.3μ/νCO	SD
Lecithin (soybean)	0.45	—	1.13	—	0.08	—	0.34	—
C_1	0.67	±0.05	1.51	±0.11	0.17	±0.02	0.19	±0.03
C_2	0.69	±0.05	1.64	±0.26	0.23	±0.02	0.30	±0.03

[a]Absorbances were calculated by the tangent base line method of Potts (13).

[b]Standard deviation. N = 4.

stretch and phosphatide P = O bend, 8.00; phosphatide C-O-C, 8.58; phosphatide C-O-C and tocopherol chroman C-O-C, 9.14; phosphatide C-O-P, 9.37; lecithin $(CH_3)_3N$, 10.3.

Analysis: Found (for adduct species C_2): C, 68.23, 68.23; H, 10.85, 10.69; P, 3.39, 3.26.

Calculated for $C_{72.1}H_{126}P_1O_{10}N_1$ (adduct of oxidized tocopherol with monomer of lecithin: C, 72.2; H, 10.6; P, 2.6.

Calculated for $C_{115}H_{204}P_2O_{18}N_2$ (adduct of oxidized tocopherol with dimer of lecithin): C, 70.3; H, 10.5; P, 3.2.

Calculated for $C_{158}H_{283}P_3O_{26}N_3$ (adduct of oxidized tocopherol with trimer of lecithin): C, 69.4; H, 10.4; P, 3.4.

Calculated for $C_{43.1}H_{78.2}P_1O_8N_1$ (average molecule of lecithin, based on our GC fatty acid analysis): C, 67.3; H, 10.2; P, 4.0.

Since the lecithin adduct species were incompletely separated from each other, they were transesterified by a mild procedure to permit characterization of the adduct moiety (12). First 9.3 mg of the lecithin adduct C_2 was dissolved in a mixture of 2 ml MeOH and 5 ml 2,2-dimethoxypropane (DMP). Then the solution was flushed 5 min with dry N_2, 0.2 ml of concentrated HCl was added and the flask stoppered. Stirring was continued at room temperature for 20 hr. Next 15 ml H_2O was added to the reaction mixture, which was extracted three times with 20 ml petroleum ether. The extract was washed twice with 10 ml H_2O, once with saturated NaCl, and dried over Na_2SO_4. After gel filtration of the extract on Sephadex LH-20 ($CHCl_3$), 3.0 mg of a pale yellow oil was eluted at 0.33 bed volumes (theoretical yield from an adduct of one tocopherol molecule with dimer lecithin, 37%;

TABLE V

Comparison of the Number Ratio of C-Methyl to Carbonyl Groups and Relative IR Absorbance for Adducts and Adduct Esters

Compound	Ratio of numbers of expected groups $C\text{-}CH_3/CO$	Observed IR absorbance ratio[a]	Relative IR absorbance ratio[e]
Lecithin	2:2 = 1.0	0.08	1.0
Proposed adduct of tocopherol with trimer of lecithin	13:6 = 2.2	0.17[b]	2.1
Proposed adduct of tocopherol with dimer of lecithin	11:4 = 2.8	0.23[c]	2.9
Lecithin fatty acid esters	1:1 = 1.0	0.044[d]	1.0
Proposed ester adduct of tocopherol and monomer of lecithin fatty acid	8:1 = 8.0	0.31	7.0
Proposed ester adduct of tocopherol and linoleic acid monomer	8:1 = 8.0	0.33	7.5

[a]$C\text{-}CH_3$ bending mode at 7.25μ and ester carbonyl at 5.72μ.

[b]Absorbance ratio for adduct C_1.

[c]Absorbance ratio for adduct C_2.

[d]Prominent absorbance at 7.34μ is from an $\alpha\text{-}CH_2$ wagging mode and not C-methyl bending (14).

[e]Absorbance ratio relative to lecithin or lecithin fatty acid ester before adduct formation.

TABLE VI

Molecular Weight and Gas Chromatography Retention Times of Adduct Esters and Reference Compounds

Compound	Mol wt	Mean relative retention time[a]	SD[b]	N[c]
Methyl oleate	296.5	1.00	---	---
Cholesterol	386.6	2.18	±0.07	3
Linoleic acid adduct ester	722.6[d]	3.65	0.02[e]	2
Lecithin adduct ester	722.6[d]	3.68	0.13	4
Cholesteryl oleate	651.0	3.79	0.15[e]	2
Triolein	885.4	4.83	0.25	4

[a]See text for conditions.

[b]Standard deviation unless specified.

[c]Number of runs.

[d]Mass spectrometry.

[e]Mean deviation of two runs.

actual yield, 32%). Simple lecithin fatty acid esters were eluted closely following the adduct esters (TLC and IR identification). TLC on Silica G (PE/EE/HAc 75:25:1) gave R_f, adduct esters, 0.79; simple fatty acid esters, 0.75.

Comparative TLC on Silica G in three other solvent systems (Table II) showed that the lecithin adduct ester had the same R_f as the linoleic acid-tocopherol adduct ester (4).

Neither the linoleic acid-tocopherol nor the lecithin-tocopherol adduct ester reacts with Emmerie-Engel spray reagents at concentrations that would give a strong positive reaction for tocopherol.

IR (μ): 5.72, ester carbonyl; 7.25(s), tocopherol methyl bend; 7.99, tocopherol aromatic ether; 8.55, ester C-O; 9.10, tocopherol chroman; small absorption at 10.1 in the region of conjugated polyene.

Hydrogenation of the adduct esters was carried out in order to remove a persistent impurity in the IR and UV spectra corresponding to ca. 1% conjugated triene. Typically, 10 mg adduct ester and 5 mg Pd (10% on charcoal) in 25 ml hexane were stirred under hydrogen at 20 psi for 3 hr. The mixture was filtered, the charcoal washed with solvent and the solvent removed at reduced pressure. Yields of a pale yellow oil are quantitative if the charcoal is carefully washed. Either the adduct ester in hexane or the lecithin adduct in $CHCl_3$ may be hydrogenated successfully. The product has a UV spectrum similar to the linoleic acid-tocopherol adduct ester (2). UV $\lambda_{max}^{CHCl_3}$ 300 nm.

The unhydrogenated adduct ester was subjected to GC on a Hewlett Packard 700 Laboratory Chromatograph modified for on-column injection and using a flame-ionization detector. Stainless steel columns (18 x 1/8 in.) were packed with 3% JXR on 100/120 mesh Gas-Chrome-Q (Applied Science Labs.). Injection port was maintained at 250 C and detector at 325 C. Carrier gas was helium at a flow rate of 60 ml/min. Temperature was programed from 100-325 C, at 10 C/min. Standard lipid reference mixtures used were K-108 (Applied Science Labs., methyl esters of fatty acids) and TLC-1 (Hormel, cholesterol, cholesterol oleate, triolein, oleic acid and methyl oleate). One μl of ester adduct solution was typically injected at a concentration of 20 μg/μl. One μl of a five-component standard mixture was injected at a total concentration of 5 μg/μl.

Adduct esters derived from lecithin appeared at an average relative retention time (RRT) of 3.68, compared to the peak for methyl esters of 18 carbon unsaturated fatty acids.

Linoleic acid-tocopherol adduct esters appeared at an average RRT of 3.65. The difference was not significant.

Peak matching mass spectrometry was performed on the unhydrogenated lecithin-tocopherol and linoleic acid-tocopherol adduct esters using a CEC Mass Spectrometer, Model 21-110B, using perfluorokerosene as internal standard as reported (2). The molecular ions for both were very prominent and for the lecithin adduct ester gave a value of 722.621, while the linoleic acid adduct ester gave 722.617, the latter confirming results reported before (2). These values are uniquely compatible with an elemental composition of $C_{48}H_{82}O_4$, based on a computer program search. The fragment ion peaks were much less prominent than those found by Gardner (6) for his addition compound, which has a simple ether linkage.

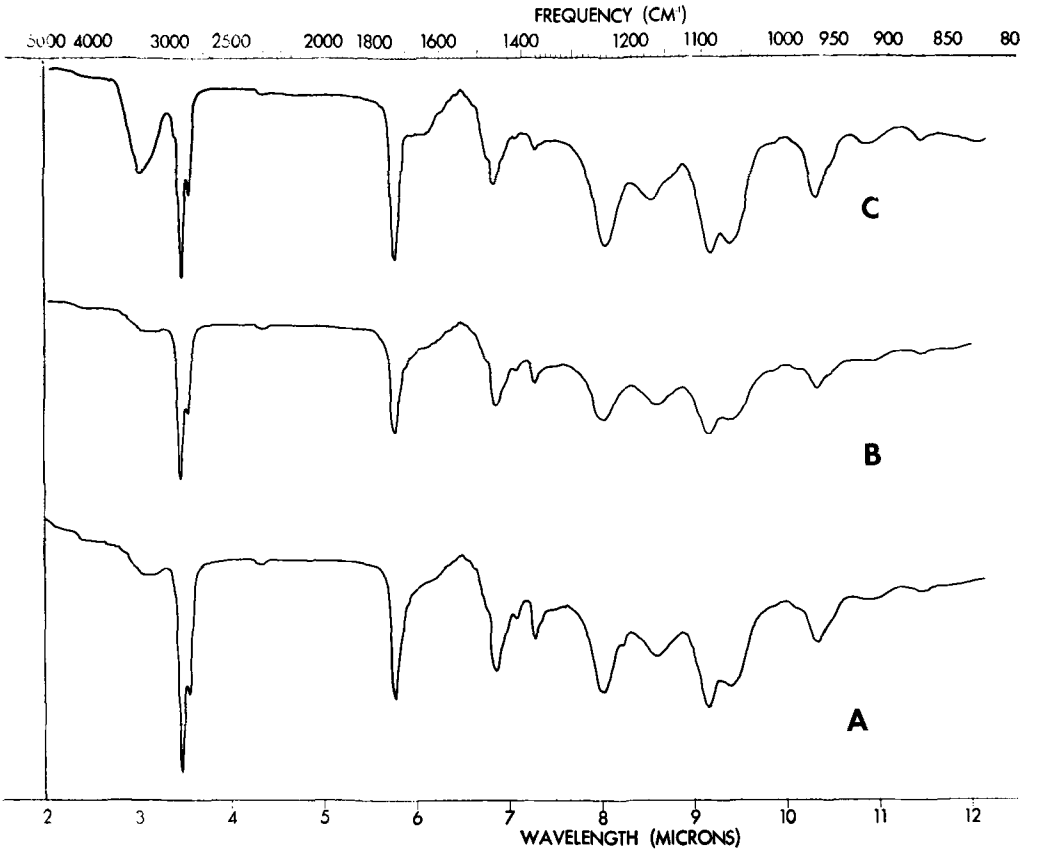

FIG. 4. IR spectra of: (A) addition compound C_2; (B) addition compound C_1; and (C) soybean lecithin. Solvent CCl_4.

RESULTS

Five 72 hr oxidation experiments were carried out with reproducible results in: (a) lecithin and tocopherol adsorption; (b) oxygen uptake; (c) silica-UV spectrophotometry; and (d) oxidation products.

Lecithin and Tocopherol Adsorption

It was found that lecithin was almost quantitatively absorbed from a chloroform solution containing both lecithin and tocopherol at concentrations of 10 mg/ml each and a total content of 200 mg each. Tocopherol, on the other hand, was largely retained in solution at equilibrium, and the amounts adsorbed in the monolayer were those contained in the ca. 2 ml trapped in the gel after decantation of supernatant. Table III shows the values for tocopherol adsorption in the five experiments, as determined by two methods. That the tocopherol aromatic moiety is actually adsorbed on the silica surface is proved by the maximum wavelength of the silica slurry spectrum prior to oxidation (8).

The mean value for tocopherol adsorption is equivalent to 7.0 mol % of the 200 mg lecithin adsorbed.

Oxygen Uptake

Mixed monolayers of soybean lecithin and tocopherol, when oxidized for 72 hr at 80 ± 5 C, showed an immediate uptake of oxygen (Fig. 1), which at 25 hr (1500 min) was ca. 29% and at 48 hr (2880 min) ca. 45% of the available oxygen. Oxygen content per flask had been planned as approximately one equivalent of the polyene fatty acids in the lecithin, the rapidly oxidizing acids. These constituted 42 wt % of the soybean lecithin by GLC assay. Under these conditions, a pure soybean lecithin monolayer takes up an equivalent of oxygen within two hours.

Except for the lack of a rapid phase, the oxygen uptake is similar to that of mixed linoleic acid and tocopherol monolayers at

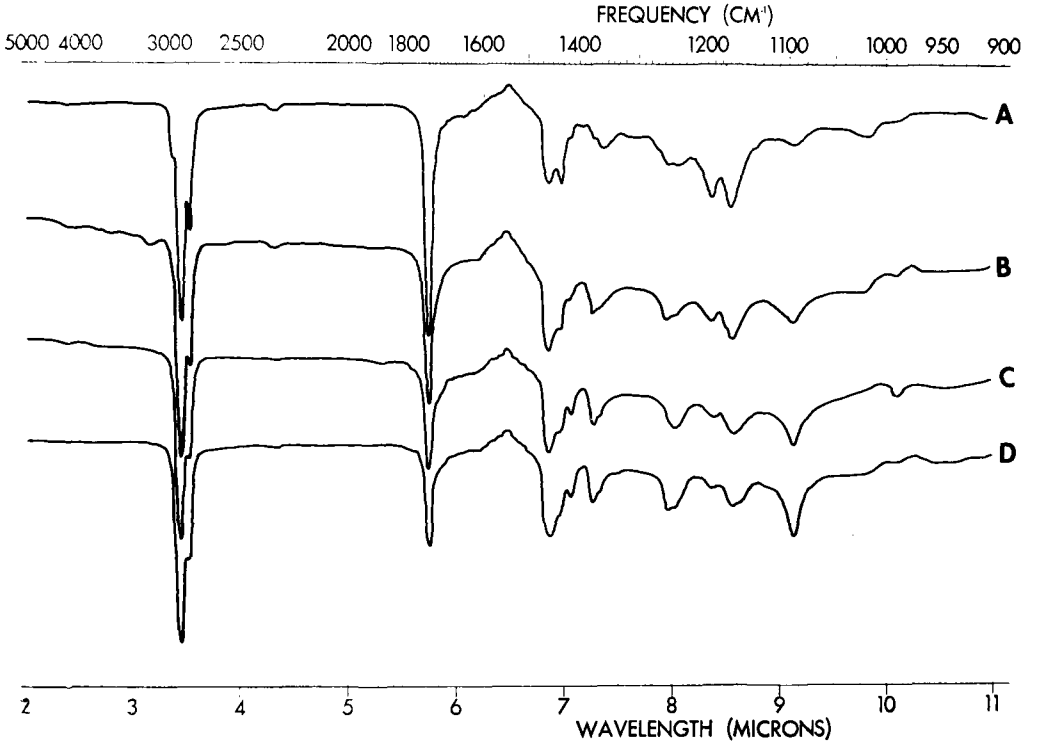

FIG. 5. IR spectra of: (A) esters of soybean lecithin fatty acids; (B) mixture of adduct esters and simple esters from transesterification of lecithin-tocopherol adduct; (C) ester of lecithin-tocopherol adduct after gel filtration; and (D) ester of linoleic acid-tocopherol adduct. Solvent CCl_4.

comparable loading ratios (2).

Silica Slurry Spectrophotometry

Silica slurry spectra (9) of oxidized monolayers of lecithin plus 7 mol % tocopherol (Fig. 2) showed no pronounced change in wavelength of maximum adsorption (λmax 287 nm) from that of the unoxidized system, 288 nm. This is the λmax of α-tocopherol on silica. There is about a two-fold increase in absorbance over the unoxidized system. Slight inflections occur in the 272 and 320 nm regions.

Since tocopherol quinones and tocopherones have molar absorptivities which are four to six times as great as tocopherol, it is clear that there has not been extensive conversion to these forms. The spectrum shows little evidence of selective absorption at the dimer wavelength (370 nm on silica [9]). Absorption at the quinone wavelength (272 nm on silica) is low. Thus the silica slurry spectrum suggests that oxidation of tocopherol in a lecithin matrix on silica produces oxidation products generally different from those reported previously by workers with other oxidants in more fluid media (1).

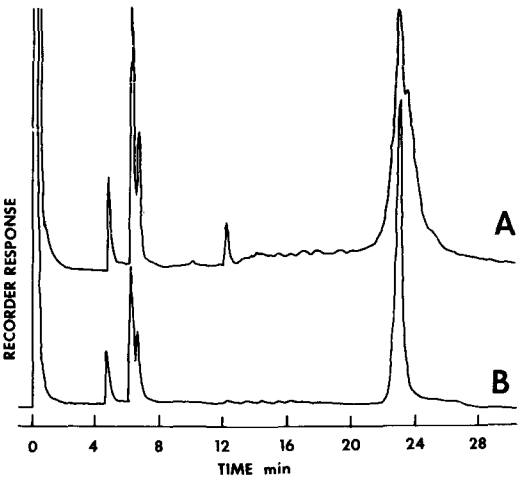

FIG. 6. Gas chromatogram of: (A) ester of lecithin-tocopherol adduct (23 min) and reference mixture of simple esters (5-7 min). Unknown peak at 12 min is an artifact of DMP esterification procedure and occurs also with simple lecithin esters (12); (B) ester of linoleic acid-tocopherol adduct and reference mixture of simple esters. See text for gas chromatography conditions.

Spectrophotometry of the Adducts in Solution

UV spectra of the two adduct species C_1 and C_2, after silica chromatography and gel filtration, were nearly identical, with $\lambda_{max}^{CHCl_3}$ 287 nm, shoulders 275, 263 and 300 nm.

The spectra have no resemblance to those of oxidation products of lecithin alone. Hydrogenation of either of the adduct mixtures or the esters of the adducts derived by transesterification removes the inflections at 263, 275 and 287 nm, leaving a peak at 300 nm (Fig. 3). The peaks removed have the same wavelength and solvent shifts as β-eleostearic acid and appear to be due to ca. 1% of a conjugated trienoic acid impurity.

IR spectra in CCl_4 of both adducts, C_1 and C_2, were highly reproducible for each of four experiments. Figure 4 shows the spectra, and Table IV some absorbance ratios of critical peaks with reference to the carbonyl stretch peak at 5.75 μ.

It is clear that in the adducts the absorbance, in the CH_2 stretch (3.4 μ) and CH_2 deformation (6.8 μ) regions and in the CH_3 deformation region (7.25 μ), is much increased relative to the carbonyl stretch absorbance as compared to the ratios in lecithin.

The 10.3 μ peak characteristic of the $(CH_3)_3N$-group of lecithin (14) is present, but its relative absorbance is less in the adducts than in lecithin.

The absorption at 9.15 μ, which is present in both the lecithin (P-O-C) and tocopherol (C-O-C) spectra, is also noticeably increased in the adducts relative to the 9.37 μ (P-O-C) peak of lecithin. The increase can be ascribed to the chroman group of tocopherol.

IR Evidence for Oligomeric Adducts

The integrated IR absorption in the C-methyl symmetric bending region (7.25 μ) has been proved dependable by Francis (15) for the determination of the number of C-methyl groups present in a compound. Although there are four types of these in the adducts, Table V shows that the relative IR absorbance in this region is compatible with a formulation of C-1 as an adduct of tocopherol with a trimer and C-2 with a dimer of lecithin. The IR absorbance of the ester adducts from either lecithin fatty acids or linoleic acid is consistent with a monomer adduct.

After transesterification and gel filtration, which separates monomers and presumed dimers of simple fatty acid esters from the adduct ester, an IR spectrum of the latter is nearly indistinguishable from that of the linoleic acid-tocopherol adduct ester (Fig. 5). The intermediate ester mixture before gel filtration has an IR whose tocopherol peaks (7.25, 9.10) are of intermediate absorbance, as expected. Figure 5 also shows the great difference between the IR of simple lecithin fatty acid esters and the adduct esters.

Gas Chromatography and Mass Spectrometry of the Adduct Esters

Figure 6 and Table VI show the GC results. The lecithin adduct ester peak is not as pure as that of the linoleic acid adduct ester, but the relative retention times of the major peak for each are not significantly different as Table VI shows. Table VI also shows that this GC liquid phase separates these compounds approximately on a molecular weight basis.

Mass spectrometry on the prominent molecular ions yields the same molecular weight and predicted elemental analysis from each of the two adduct esters derived from different sources. Thus the two adduct esters are indistinguishable by all chemical, chromatographic and spectral evidence so far available. These data confirm the IR evidence that the adduct esters are 1:1 addition compounds.

DISCUSSION

The above data are consistent with formation of adducts of oxidized tocopherol with the fatty acid moieties of intact lecithin, as has been shown for linoleic acid (2).

The results of both the IR and the elemental analysis of the lecithin adduct C_2 (before transesterification) reveal a CH_3 absorbance and a phosphorus content which are, respectively, too low and too high for a simple one-to-one adduct of lecithin and tocopherol. They are, however, more consistent with a formulation as an adduct of oxidized tocopherol with a dimer or trimer of lecithin, the tocopherol moiety having added to one of the fatty acids not involved in the polymerization. Thus the ester adduct freed by transesterification would be identical by all tests so far used with that from linoleic acid and tocopherol.

The most likely intermediate in the presumed radical addition is a transitory ortho-quinone methide species similar to that suggested for the tocopherol dimer (1,3,16). Sprengling (17) suggested such a quinone methide in the reaction of an *o*-hydroxymethyl phenol and oleic acid at 180 C and under CO_2 to give a chroman ring adduct analogous to that suggested here.

ACKNOWLEDGMENTS

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Elemental analysis of lecithin adducts was performed by S. Nagy.

REFERENCES

1. Skinner, W.A., and R.M. Parkhurst, Lipids 6:240 (1971).
2. Porter, W.L., L.A. Levasseur and A.S. Henick, Ibid. 6:1 (1971).
3. Nilsson, J.L.G., J.O. Branstad and H. Sievertsson, Acta. Pharm. Suecica 5:509 (1968).
4. Skinner, W.A., and R.M. Parkhurst, J. Org. Chem. 31:1248 (1966).
5. Komoda, M., and I. Harada, JAOCS 47:249 (1970).
6. Gardner, H.W., K. Eskins, G.W. Grams and G.E. Inglett, Lipids 7:324 (1972).
7. Porter, W.L., L.A. Levesseur, J.E. Jeffers and A.S. Henick, Ibid. 6:16 (1971).
8. Singleton, W.S., M.S. Gray, M.L. Brown and J.L. White, JAOCS 42:53 (1965).
9. Porter, W.L., A.S. Henick, J.I. Jeffers and L.A. Levasseur, Lipids 6:9 (1971).
10. Parker, W.E., and W.D. McFarlane, Can. J. Res. 18B: 405 (1940).
11. Skinner, W.A., and P. Alaupovic, J. Org. Chem. 28:2854 (1963).
12. Radin, N.S., A.K. Hajra and K. Akahari, J. Lipid Res. 1:250 (1960).
13. Potts, W.J., "Chemical Infrared Spectroscopy," John Wiley & Sons, Inc., New York, 1963, p. 168.
14. Chapman, D., "The Structure of Lipids by Spectroscopic and X-Ray Techniques," John Wiley & Sons, Inc., New York, 1965, p. 113.
15. Francis, S.A., J. Chem. Phys. 18:861 (1950).
16. Turner, A.B., Quart. Rev. Chem. 18:347 (1964).
17. Sprengling, G.R., J. Amer. Chem. Soc. 74:2937 (1952).

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Effect of Soy Sterols on Cholesterol Synthesis in the Rat

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ABSTRACT

Soy sterols increased the incorporation of acetate into cholesterol by rat liver slices and prevented the depression of cholesterogenesis caused by dietary cholesterol. Acetate incorporation into cholesterol by intestinal slices was not affected by soy sterols but was decreased by cholesterol. The results with liver slices agree with the view that soy sterols, by interfering with the intestinal absorption of cholesterol, prevent the feedback inhibition of hepatic cholesterogenesis.

INTRODUCTION

Dietary plant sterols, given in large amounts, prevent the increase in tissue cholesterol caused by ingestion of cholesterol, as first reported by Peterson (1) for chicks, and confirmed since then for many other species. This effect has been ascribed to interference with cholesterol absorption, but recent studies with lymph-fistulated rats have failed to reveal any effect of plant sterols on cholesterol absorption (2,3). Konlande and Fisher (4) reported that parenterally administered phytosterols had a cholesterol-depressing effect in chicks and postulated a nonabsorptive action on cholesterol metabolism.

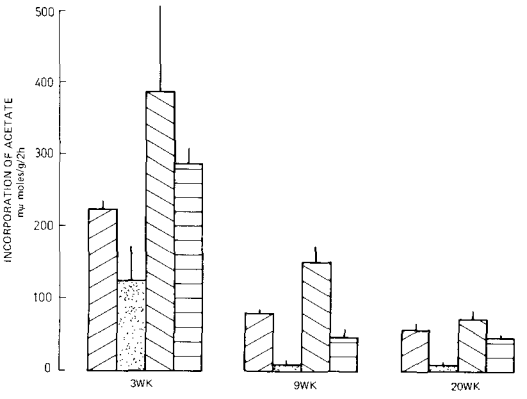

FIG. 1. Effect of dietary sterol supplements on hepatic sterol synthesis in rats of different ages. Supplements were given for 8 days. Height of columns represents means for five rats; vertical lines at top of columns are standard errors of the mean. ▨ No supplement; ▩ cholesterol 1%; ▧ soy sterols 2%; and ▤ cholesterol 1% + soy sterols 2%.

The purpose of this study was to examine the effect of dietary soy sterols on hepatic cholesterogenesis and to interpret the results in the light of the negative feedback mechanism operating in the liver. Intestinal sterol synthesis was also investigated.

MATERIALS AND METHODS

Male Wistar rats were maintained on a balanced stock diet (Ambar Ltd., Hadera, Israel) and were given the experimental diets at the ages indicated, for a period of 8 days. The experimental diets were prepared by adding 5% oleic acid and the sterol supplements to the ground stock diet. The sterols were first dispersed in the oleic acid by heating, and the oily mixture was then added to the stock diet while still warm. Mixed soy sterols contained 89% sterols and ca. 5% steryl esters. Gas liquid chromatographic analysis showed that 54% of the total sterols were β-sitosterol, 25% campesterol and 22% stigmasterol. The gas liquid chromatographic results and procedural details for the analysis of the mixed soy sterols were given in a previous paper from this laboratory (5).

Rats were killed by a blow on the head, and livers and distal ileal halves were excised. Preparation of slices and measurement of incorporation of Na 1-^{14}C-acetate into digitonin-precipitable sterols (DPS) were as described by Dietschy and Siperstein (6), using 500 mg samples and 1 μCi of Na 1-^{14}C acetate (The Radiochemical Centre, Amersham; specific activity: 62 mCi/mmol). Incorporation, determined in this manner, is not specific for cholesterol, and strictly speaking, "sterogenesis," rather than "cholesterogenesis," was measured. Nevertheless, with this qualification in mind and in view of the vast body of evidence concerning the incorporation of acetate into cholesterol, we have used the above terms interchangeably.

Radioactivity was measured in a TriCarb Liquid Scintillation Counter. Cholesterol was determined colorimetrically as described by Katz et al. (5).

RESULTS AND DISCUSSION

The results of a feeding test carried out on rats aged 9 weeks are presented in Table I. The

TABLE 1

Effect of Dietary Sterol Supplements on Hepatic and Intestinal Sterol Synthesis in Vitro[a]

Supplements	Liver		Intestine	
	DPS content, mg/g	Acetate incorporation, mμmoles/g/2hr	DPS content, mg/g	Acetate incorporation, mμmoles/g/2hr
None (control)	3.47 ± 0.12	79.9 ± 6.1	3.03 ± 0.14	70.5 ± 8.1
Cholesterol 1%	5.52 ± 0.43[b]	10.3 ± 2.6[b]	2.81 ± 0.26	40.5 ± 14.1[b]
Soy sterols 2%	3.17 ± 0.54	149.6 ± 21.6[b]	4.04 ± 0.61[c]	66.4 ± 8.0
Cholesterol 1% + soy sterols 2%	3.54 ± 0.47	46.8 ± 11.4[b]	3.21 ± 0.14	29.3 ± 7.8[b]

[a]Rats weighing ca. 192 g were given the supplements for 8 days. The daily food intake was 21 g per rat, without significant differences between treatments. Figures are means and standard errors for 11 livers and 9 intestines.

[b]Highly significantly different from control value ($P<0.01$).

[c]Significantly different from control value ($P<0.05$).

results illustrate the well known inhibitory effect of dietary cholesterol on hepatic cholesterogenesis and also show that this effect was partly offset by the simultaneous administration of soy sterols. On the other hand, dietary supplementation with soy sterols alone caused an increase in sterogenesis to nearly twice the value of the controls. The opposite effects of cholesterol and soy sterols were also reflected, in a less striking fashion, in the hepatic sterol concentrations, with the soy sterols counteracting the increase induced by dietary cholesterol. Table I also shows that the sterogenic response of ileal slices to dietary sterol supplementation differed from that of the liver; cholesterol feeding again caused a significant decrease in acetate incorporation, but soy sterols, fed alone, were without effect and did not prevent the inhibition due to dietary cholesterol.

Incubation experiments similar to the above were carried out with liver slices from rats of different ages (Fig. 1). Although the sterogenic capacity of the liver decreased with age, soy sterols always caused enhanced acetate incorporation into sterols and also partially or completely suppressed the inhibitory action of dietary cholesterol.

The observed effects of soy sterols on hepatic cholesterogenesis in vitro are consistent with the generally held view according to which plant sterols interfere with the normal absorption of cholesterol: this interference should offset the pronounced negative feedback inhibition in cholesterol-fed animals. Moreover, interruption of the enterohepatic circulation of endogenous cholesterol in rats on a low cholesterol diet would be expected to cause an increase in hepatic cholesterogenesis above that of control animals. The effect of soy sterols on hepatic sterol synthesis in rats observed in the present study, is similar to that reported by Cayen (7) for tomatine, which forms a non-absorbable complex with cholesterol. Grundy et al. (8), on the basis of sterol balance experiments with humans, concluded that ingestion of large amounts of plant sterols caused increased synthesis of cholesterol.

The pronounced inhibition of intestinal sterogenesis by dietary cholesterol indicates that a negative feedback also operates in the intestinal cells. Dietschy (9), in reporting a similar cholesterol effect earlier, implicated the increased formation and enterohepatic circulation of bile acids, which are known to suppress cholesterol synthesis in the intestine. However the lack of effect of soy sterols is difficult to reconcile with this view, since the interruption of the exogenous cholesterol supply to the liver by soy sterols would be expected to re-establish normal hepatic bile acid synthesis and normal cholesterogenesis, which is clearly not the case. The possible direct involvement of cholesterol in the regulation of intestinal cholesterogenesis was recently envisaged by Cayen (7), who reported increased synthesis of cholesterol in the intestine of tomatine-fed rats, with no change in bile acid excretion.

While the interpretation of the results obtained with intestinal slices hinges on a better understanding of the regulation of intestinal sterol synthesis and the nature of the interaction between cholesterol and plant sterols, the liver results obtained in the present study lend support to the view that soy sterols decrease the absorption of endogenous cholesterol from the intestine, thus causing the release of the negative feedback control of hepatic

cholesterol synthesis.

ACKNOWLEDGMENT

Mixed soy sterols were donated by N. Embree, Tennessee Eastman Co., Kingsport, Tenn. After the paper was submitted for publication, the authors received a personal communication from M.N. Cayen, stating that 2% β-sitosterol enhanced cholesterogenesis in livers, but not in intestinal sections, in 2 week feeding tests with male rats.

REFERENCES

1. Peterson, D.W., Proc. Soc. Exp. Biol. Med. 78:143 (1951).
2. Sylvén, C., and B. Borgström, J. Lipid 10:179 (1969).
3. Klauda, H.C., and F.M. Quackenbush, Li 5:142 (1970).
4. Konlande, J.E., and H. Fisher, J. Nutr. 98 (1969).
5. Katz, M., I. Bartov, P. Budowski and A. Bon Nutr. 98:1141 (1970).
6. Dietschy, J.M., and M.D. Siperstein, J. Lipic 8:97 (1967).
7. Cayen, M.N., J. Lipid Res. 12:482 (1971).
8. Grundy, S.M., E.H. Ahrens, Jr. and J. Davi Ibid. 10:304 (1969).
9. Dietschy, J.M., J. Clin. Invest. 47:286 (1968

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SHORT COMMUNICATIONS

Effects of Parathion on Lipolysis in Isolated Rat Adipose Tissue Cells[1]

ABSTRACT

The effects of the pesticide, Parathion (0,0-diethyl 0-*p*-nitrophenyl thiophosphate) and various lipolytic and antilipolytic agents on lipolysis in adipose tissue were studied with isolated fat cells from rat epididymal fat pads. Lipolysis was measured as the release of free fatty acids into an albumin-bicarbonate medium. Parathion depressed lipolysis in a linear manner at concentrations ranging from 10^{-9} to 10^{-3}M. At a concentration of 10^{-5}M, Parathion depressed the lipolytic response to epinephrine (0.15 μg/ml) and slightly to cyclic 3',5'-adenosine monophosphate (10^{-3}M) and enhanced the antilipolytic response to nicotinic acid (33 μM). It is possible that Parathion may interfere with adenyl cyclase activity.

INTRODUCTION

The development of the isolated fat cell technique (1) has prompted considerable research into the mechanism of lipolysis in adipose tissue as well as into the site of action of a spectrum of lipolytic and antilipolytic substances (2-9). Catecholamines and peptide hormones act upon adenyl cyclase, which increases cyclic AMP. This in turn activates the lipase. Nicotinic acid stimulates phosphodiesterase hydrolyzing cyclic AMP with a resultant decrease in lipolytic activity. The two enzymes, adenyl cyclase and phosphodiesterase, are thus key factors in controlling lipolysis. In this paper evidence is presented for the inhibition of lipolysis in isolated rat adipose tissue cells by the pesticide, Parathion.

MATERIALS AND METHODS

The adipose tissue was obtained from the thin distal portions of the epididymal fat pads from male Wistar rats (150-220 g). These were placed in cold (4 C) isotonic saline. The right pad was routinely used for experimental trials and the left pad for controls. Isolated fat cells were prepared essentially according to the procedure of Rodbell (1). Half the stated concentration of calcium was used in these experiments.

The isolated fat cells were distributed with disposable plastic pipets (Biopette, Schwarz), 0.1 ml per polyethylene vial containing 1.4 ml Krebs bicarbonate-albumin buffer, pH 7.4 gassed with 95% air -5% CO_2 prior to use. Ethanol was employed as a carrier to aid in the solution of Parathion in the aqueous medium. The ethanol-Parathion solution was sonicated in the albumin buffer to assist dispersion. Ethanol was also added to the basal-lipolysis control vials. The final concentration of ethanol was

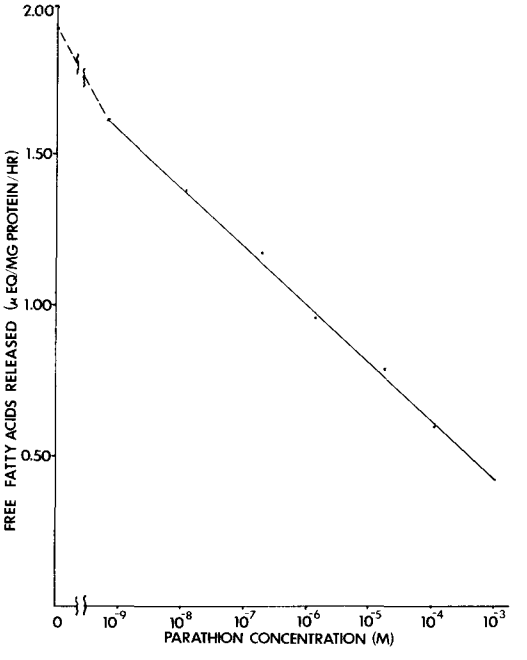

FIG. 1. Inhibition of lipolysis in isolated rat adipose cells by Parathion.

[1]Scientific Contribution No. 400. Agricultural Experiment Station, University of Connecticut, Storrs, Conn. 06268.

3%. Three trials were done at each level of Parathion.

Incubations were carried out for 60 min in a water bath shaker at 160 rpm at 37 C with the vials capped. Parathion or nicotinic acid was added before the distribution of cells in the medium. Activators of lipolysis, epinephrine (Mann) or cyclic AMP (Nutritional Biochemicals) were added to the fat cell suspension after 30 min of incubation.

Incubation was terminated by transferring 0.5 ml of the fat cell suspension to $CHCl_3$ or 5% TCA for fatty acid and protein determination, respectively. Free fatty acid release was determined by the method of Duncombe (10) and protein by the procedure of Cleland and Slater (11) as modified by Blecher et al. (4). The protein bound triglyceride was saponified with 1 N NaOH at 100 C instead of 0.1 N NaOH (11) for 3 min, because of the high fat concentration in the cell preparations. Palmitic acid (Hormel) and bovine serum ambumin (Calbiochem) were routinely employed as standards.

RESULTS AND DISCUSSION

The inhibitory effect of Parathion on lipolysis is depicted in Figure 1. The inhibition was linear, increasing as the concentration of Parathion was raised and, in general, confirms the results of others noted below.

Several other experiments were done to study the interaction of Parathion with known stimulators and inhibitors of lipolysis. The results were as follows: (name of drug, concentration used and rate of lipolysis expressed as μ Eq of free fatty acids/mg protein/hr underlined.) *Experiment 1:* Control, 3.1; epinephrine, 0.15 mg/ml, 9.2; epinephrine, 0.15 mg/ml + Parathion, 10^{-5}M, 5.1. *Experiment 2:* Control 0.7; cAMP, 10^{-3}M, 1.9; cAMP, 10^{-3}M + Parathion, 10^{-5}M, 1.7. *Experiment 3:* Control, 2.0; Parathion, 10^{-5}M, 0.9; nicotinic acid, 33 μM, 1.1; nicotinic acid, 33 μM + Parathion, 10^{-5}M, 0.6.

Since Parathion inhibited the action of epinephrine on lipase activity but not the effect of cAMP, our data suggest that Parathion acted at the level of adenyl cyclase rather than directly on the lipase. The action of Parathion at the site of the first messenger (epinephrine) rather than at that of the second messenger (cAMP) is consistent with the extremely low concentrations of Parathion required to inhibit lipolysis.

However other evidence suggests that Parathion and other organophosphates inhibit lipases directly. Parathion was reported to be an inhibitor of partially purified pancreatic, *Geotrichum candidum* and *Vernonia anthelmintica* lipases (12). Several similar organophosphates reduced the activity of rat heart diolein hydrolase and rat brain acetylcholine esterase (13). Paraoxon (diethyl-*p*-nitrophenylphosphate, 10^{-6}M) inhibited lipolytic activity in eggs from the southern root cornworm (14). The organophosphates may bind the enzymes at a serine hydroxyl residue as they do with acetylcholine esterase (15).

If further studies establish the site of action of Parathion at the level of adenyl cyclase, this drug may prove useful in elucidating the mechanism of action of various lipolytic agents.

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REFERENCES

1. Rodbell, M., J. Biol. Chem. 239:375 (1964).
2. Mosinger, B., and M. Vaughn, Biochim. Biophys. Acta 144:569 (1967).
3. Butcher, R.W., C.E. Baird and E.W. Sutherland, J. Biol. Chem. 243:1713 (1968).
4. Blecher, M., N.S. Merlino and J.T. Ro'Anne, Ibid. 243:3973 (1968).
5. Birmbaumer, L., and M. Rodbell, Ibid. 244:3477 (1969).
6. Hepp, D., D.R. Challoner and R.H. Williams, Ibid. 243:4020 (1968).
7. Butcher, R.W., and C.E. Baird, Ibid. 243:1713 (1968).
8. Krishna, G., B. Weiss, J.I. Davies and S. Hynie, Fed. Proc. 25:719 (1966).
9. Vaughan, M., J.E. Berger and D. Steinberg, J. Biol. Chem. 239:401 (1964).
10. Duncombe, W.G., Biochem. J. 83:6P (1962).
11. Cleland, K.W., and E.C. Slater, Ibid. 53:547 (1953).
12. Carpenter, D.L., and R.G. Jensen, Agr. Exp. Sta. Bull. (Storrs, Conn.) 1971:414.
13. Buchet, V.P., and R. Lauwerys, Biochim. Biophys. Acta 218:369 (1970).
14. Krysan, J.L., and P.L. Guss, Ibid. 239:349 (1971).
15. O'Brien, R.D., "Toxic Phosphorous Esters," Academic Press, New York, 1960, p. 175ff.

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Effect of Double Bond Position in Octadecenoates upon Hydrolysis by Pancreatic Lipase[1]

ABSTRACT

Fifteen triacylglycerols containing 12:0, 14:0, 16:0, 18:2 and one positional isomer of *cis*-18:1 were hydrolyzed by pancreatic lipase (EC 3.1.1.3, glycerol ester hydrolase). The fatty acids in the products of lipolysis were identified and measured by gas liquid chromatography. The substrates containing the Δ2 through Δ7 isomers of 18:1 were resistant to pancreatic lipolysis. These isomers accumulated in the di- and residual triacylglycerols and were diminished in the free fatty acids. The discrimination was greatest against the Δ5 isomer.

INTRODUCTION

A double bond near the carboxyl ester in straight chain fatty acid esterified to glycerol at a primary hydroxyl has been found to inhibit the activity of pancreatic lipase. Bottino et al. (1) noted that eicosapentaenoate and docosahexaenoate resisted pancreatic lipolysis. They attributed the resistance to the location of a *cis* double bond near the esterified carboxyl group or to the many *cis* double bonds in the chain placing the terminal methyl group in a position near the ester bond, thereby causing steric hindrance. Kleiman et al. (2) observed that esters of *trans*-3-enoic acids in various seed oils were resistant to pancreatic lipolysis. Mattson and Volpenhein (3) similarly found that *cis*-6-octadecenoate resisted lipolysis. Brockerhoff (4), observing resistance of esters of both *cis* and *trans* unsaturated acids to pancreatic lipase, postulated that the inhibition was due to steric hindrance during the formation of the activated complex. However there has been no systematic investigation of the effects of positional isomerism in long chain unsaturated fatty acids on pancreatic lipase activity. We have recently completed a study of the specificity for *cis*-9-18:1 of the lipase from the microorganism *Geotrichum candidum* in which individual triacylglycerols containing one each of the positional isomers of *cis*-18:1 were used as substrates (5). Each of these fifteen triacylglycerols, which were nearly random mixtures of triglycerides 12:0, 14:0, 16:0, 18:2 and one positional isomer of 18:1, was also hydrolyzed by pancreatic lipase. The products of lipolysis were recovered and the fatty acids identified. The results are reported herein.

METHODS

The procedures for incubations have been described in detail (5). They were modified slightly, in that the amount of porcine pancreatic lipase (Mann Biochemical, extracted three times with diethyl ether) was 30 mg and the length of lipolysis was reduced to 10 min. The products of hydrolysis and the residual triglyceride were analyzed by gas liquid chromatography, and the composition of each fraction, with respect to the 18:1 and other acids, indicated whether there was discrimination of fatty acids by the enzyme. The compositions, reported as mole per cents, were normalized with respect to an internal standard of 17:0, a constant amount of which was added to each fraction before conversion to methyl esters. The quantity of 17:0 was set at unity, and the resultant values calculated as mole per cents are therefore comparable between substrates. The degree of hydrolysis was 50-60% in all cases.

RESULTS AND DISCUSSION

If a double bond near a carboxyl group of an esterified acid inhibits the activity of the

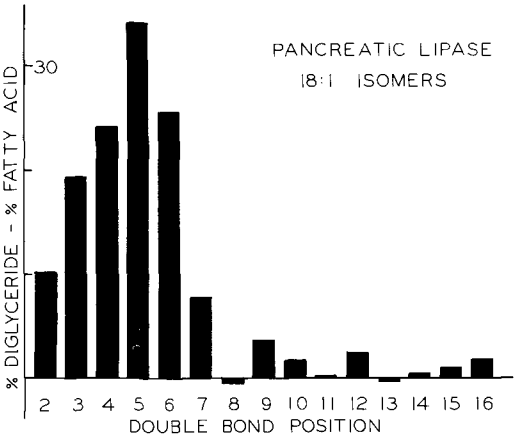

FIG. 1. The degree of discrimination against isomeric *cis* 18:1 acids present in random mixed triacylglycerols, each of which contained one isomer. Ordinate values are the per cent of isomeric 18:1 in diacylglycerols minus the per cent of isomeric 18:1 in free fatty acids. If an acid is neither discriminated for nor against, the difference should be zero.

[1]Scientific Contribution No. 504. Agricultural Experiment Station, University of Connecticut, Storrs, Conn. 06268.

TABLE I

Content (mol %)[a] of Isomeric *cis* 18:1 Acids in Esters Formed from Products of Hydrolysis of Random Mixed Triglycerides after Action by Pancreatic Lipase

Positional isomer	Fatty acid	Monoglyceride	Diglyceride	Residual triglyceride	Original triglyceride
2	0	4.5	10.2	3.8	3.6
3	6.2	9.9	25.5	11.2	9.2
4	17.3	23.2	41.4	24.6	23.4
5	12.2	35.0	45.3	29.5	23.9
6	14.2	20.9	39.7	18.5	22.2
7	17.2	21.5	24.9	22.4	22.5
8	21.8	21.9	21.3	26.0	22.8
9	20.1	19.6	23.7	20.5	22.1
10	20.0	23.7	21.7	22.1	26.4
11	21.4	19.9	21.6	20.2	22.5
12	18.6	20.8	21.0	21.7	22.6
13	20.9	20.9	20.6	21.7	21.9
14	16.9	18.2	17.3	18.7	18.9
15	23.0	24.4	24.0	25.9	25.1
16	13.3	14.4	15.1	15.2	14.9

[a]Refers to the content of each isomer in the total fatty acids after hydrolysis of MG, DG or TG.

enzyme, that fatty acid should therefore occur in the diacylglycerol and monoacylglycerol products in higher proportion than in the original triacylglycerols, and in lower proportion in the liberated fatty acids. The contrast should be greatest between its content in the initial fatty acids hydrolyzed, and the initial residual product, the diacylglycerols. The compositions (mol %) of the products of hydrolysis, with respect to the 18:1 isomers, are listed in Table I. The accumulation of Δ2 through Δ7-18:1 isomers in the residual triacylglycerols was not as marked as expected, with only the Δ-5 showing any appreciable difference—29.5 compared to 23.9 mol % in the original substrate. The 18:2 content of the residual triacylglycerols was less in every case than in the original triacylglycerols, often less than half, suggesting discrimination during lipolysis or perhaps relatively slower acylation by 18:2 of secondary hydroxyls during synthesis, or both.

In Table I the contents of Δ2 through Δ7 isomers in the hydrolyzed acids were lower in every case than those in the mono-, di- and residual triacylglycerol products of lipolysis, indicating resistance to lipolysis. The proportions of Δ8 through Δ16 isomers in fatty acids and in the other products were about the same, indicating no discrimination against these isomers.

The diacylglycerols contained relatively large amounts of the Δ2-6 isomers. These data are the most striking indication of inhibition by a *cis* double bond in proximity to the carboxyl-ester.

Figure 1 shows the per cent of each isomer in the diacylglycerols minus the per cent of isomer in the free fatty acids. These data indicate the degree of discrimination against hydrolysis of the monoenoic acids. In those isomers in which the double bond is close to the carboxyl group, the hydrolysis of the isomer was inhibited. These results confirm and extend the observations that a double bond near the carboxyl group of an acid inhibits its hydrolysis from a triacylglycerol (1-3). The discrimination is greatest against the Δ5 isomer in the 18:1 series, and when the double bond is beyond carbon 7 the discrimination approaches experimental error.

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REFERENCES

1. Bottino, N.R., G.A. Vandenberg and R. Reiser,

Lipids 2:489 (1967).
2. Kleiman, R., F.R. Earle, W.H. Tallent and I.A. Wolff, Ibid. 5:513 (1970).
3. Mattson, F.H., and R.A. Volpenhein, AACC-AOCS Joint Meeting, Washington, D.C., April 1968.
4. Brockerhoff, H., Biochim. Biophys. Acta 212:92 (1970).
5. Jensen, R.G., D.T. Gordon, R.T. Holman and W.H. Heimermann, Lipids 7:738 (1972).

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Sulfated Bile Acid in Urine of Patients with Hepatobiliary Diseases

ABSTRACT

The presence of sulfated bile acid in urine of patients with hepatobiliary diseases was recognized by using an Amberlite XAD-2 column for extraction of bile acid and a Sephadex LH-20 column for separation of sulfated (sulfate of either taurine or glycine conjugate) and nonsulfated bile acid (taurine and glycine conjugate). Sulfated and nonsulfated bile acid, obtained after a Sephadex LH-20 column of urinary extract of the patient with acute hepatitis, was identified by thin layer chromatography. Sulfated bile acid showed a spot with different Rf value from that of taurine-conjugated, glycine-conjugated and free bile acid, and solvolysis of sulfated bile acid resulted in a compound with the same Rf value as glycodihydroxycholanoic acid. A large amount of bile acid sulfate was found in urine of patients with hepatobiliary diseases. The sulfated bile acid in these urine samples occupied from 57.1 to 93.3% of total bile acid, and consisted of both di- and trihydroxycholanoic acid (major part, chenodeoxycholic acid). As no solvolysis was carried out in previous works, bile acid sulfate in urine, as described in this paper, was not determined at all.

It is well known that concentration of serum bile acid increases, and large amounts of bile acid are excreted into urine of patients with hepatobiliary diseases (1-6). However the information on bile acid metabolism in previous papers has been restricted to aspects of taurine- and glycine-conjugated and unconjugated bile acid, and little work has been done about other forms of bile acid conjugates in human biological materials (7,8).

In the course of our studies on bile acid metabolism, we found the presence of a new form of bile acid conjugate, sulfated taurine or glycine-conjugated bile acid, in urine and serum of patients with hepatobiliary diseases.

In this paper the presence of sulfated bile acid in urine of patients with hepatobiliary

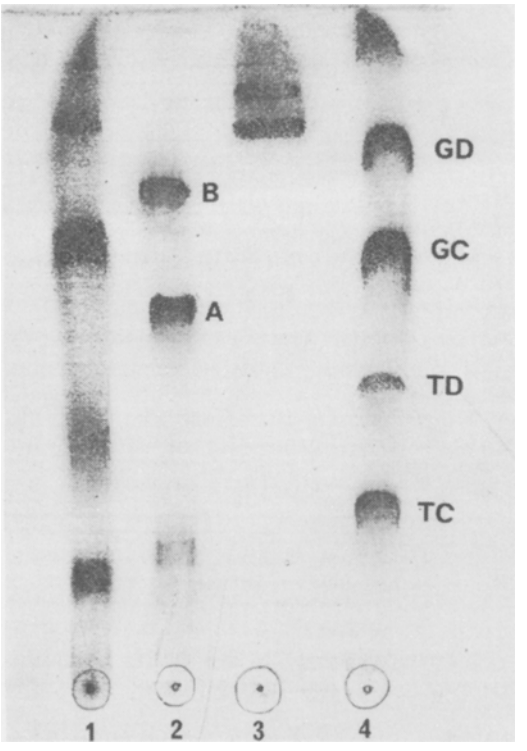

FIG. 1. Thin layer chromatography of nonsulfated and sulfated bile acid in urine of the patient S.S. 1. Nonsulfate fraction; 2. sulfate fraction; 3. sulfate fraction after solvolysis; 4. Standard bile acids. GD = Glycodihydroxycholanoic acid; GC = glycocholic acid; TD = taurodihydroxycholanoic acid; and TC = taurocholic acid. Silica Gel G provided the layer. Solvent system was *n*-butanol-acetic acid-water 10:1:1. Sulphuric acid was the spray.

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In the course of our studies on bile acid metabolism, we found the presence of a new form of bile acid conjugate, sulfated taurine or glycine-conjugated bile acid, in urine and serum of patients with hepatobiliary diseases.

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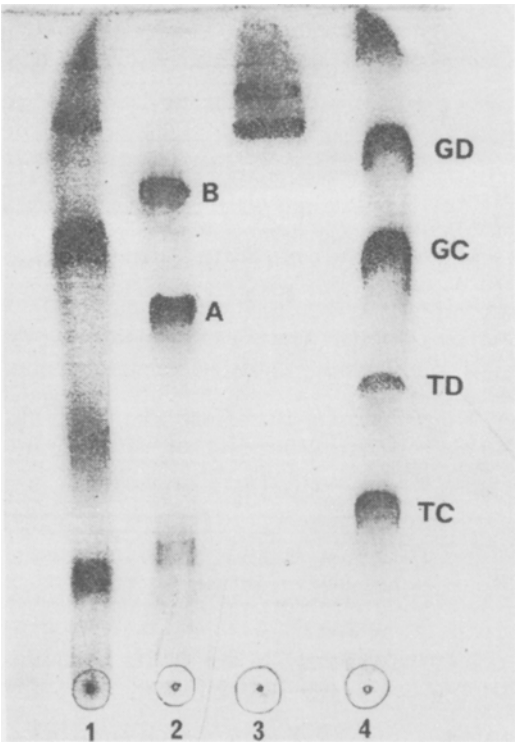

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TABLE I

Daily Excretion of Nonsulfated and Sulfated Bile Acid in Urine of Patients with Hepatobiliary Diseases

	Patient N.K. (male, 51 years)	Patient Y.I. (male, 53 years)	Patient S.S. (female, 27 years)	
	Carcinoma of gallbladder	Carcinoma of Papilla Vater	Acute hepatitis	
Excretion of bile acid in urine, mg/24 hr				
Nonsulfated bile acid				
Deoxycholic	(-)	(-)	0.03	
Chenodeoxycholic	0.06	0.14	1.08	
Cholic	2.34	5.53	2.97	
Total	2.40	5.67	4.08	
Sulfated bile acid				
Deoxycholic	(-)	(-)	1.33	
Chenodeoxycholic	4.79	6.21	53.53	
Cholic	4.06	1.33	2.16	
Total	8.85	7.54	57.02	
Total bile acid	11.25	13.21	61.10	
Per cent of sulfated to total bile acid	78.7	57.1	93.3	
Liver function test[a]				Normal
Kunkel, units	12.3	3.5	6.1	(3-10)
T. bilir., mg/dl	22.9	27.0	14.5	(0.3-1.0)
Al-Ph, units	19.1	76.0	9.4	(2.7-10.0)
SGOT, units	78	147	348	(5-40)
SGPT, units	50	88	243	(3-35)

[a]T. bilir. = total serum bilirubin concentration; Al-Ph = serum alkaline phosphatase (in King-Armstrong units); SGOT = serum glutamic oxalacetic transaminase; and SGPT = serum glutamic pyruvic transaminase.

diseases is described.

The three patients with carcinoma of gallbladder (N.K.), carcinoma of Papilla Vater (Y.I.) and acute hepatitis (S.S.) were studied. Thirty milliliters of urine was applied to an Amberlite XAD-2 column which retained steroids and bile acids (9). The retained substances were eluted with methanol, and after evaporation to dryness the residue was applied on a Sephadex LH-20 column. The nonsulfate fraction of the glycine and taurine conjugates was eluted with 70 ml chloroform-methanol 1:1 v/v containing sodium chloride (0.01 mol/liter), and the sulfate fraction of either taurine or glycine conjugate was then eluted with 50 ml methanol (10-12).

The sulfate fraction was subjected to solvolysis in 30 ml ethanol-acetone 1:9 v/v acidified with a few drops of 2N HCl to pH 1 for 2 days (8), saponified, then extracted with ether after acidification and methylation. The resulting materials were purified on an aluminum oxide column and analyzed by gas chromatography using a QF-1 column after conversion into the trifluoroacetate derivatives of the methyl esters (3).

For identification of sulfated bile acid, an aliquot of two fractions (the sulfate and the nonsulfate fractions), obtained after separation on a Sephadex LH-20 column of the urinary extract of the patient S.S., was analyzed by thin layer chromatography (Silica Gel G) using *n*-butanol-acetic acid-water 10:1:1 as solvent system (13). The thin layer chromatograms of the sulfate and the nonsulfate fractions are shown in Figure 1. A large spot corresponding to standard glycocholic acid was observed for the nonsulfate fraction, while two spots (A and B) with different Rf values from those of taurine and glycine conjugates were detected for the sulfate fraction. The materials in the zones corresponding to spots A and B were scraped off and eluted with methanol. The eluates were subjected to solvolysis, alkaline hydrolysis, methylation and trifluoroacetylation without purification on an aluminum oxide column, and then analyzed by gas chromatography. The area A contained dihydroxycholanoic acid (major part, chenodeoxycholic acid; minor part, deoxycholic acid), while no bile acid was observed in the area B. Solvolysis of the sulfate fraction resulted in a compound with the same Rf value as glycodihydroxycholanoic acid (Fig. 1).

According to these results, the spot A seems to be the sulfated glycodihydroxycholanoic acid that was more polar than original glycodihydroxycholanoic acid.

Table I gives the results of quantitative estimation of bile acid sulfate in urine of three

patients with hepatobiliary diseases. The percentages of bile acid sulfate to total bile acid in urine were 78.7, 57.1 and 93.3, respectively, and these ratios were unexpectedly high. The major sulfated bile acid was derived from dihydroxycholanoic acid, especially chenodeoxycholic acid, while the major bile acid of the nonsulfated fraction was cholic acid.

Recently we reported the presence of sulfated bile acid in human serum (7), but its presence in human urine has not been reported. It was therefore surprising to find a large amount of sulfated bile acid in urine as described in this paper.

The sulfated bile acid fraction was obtained after the Sephadex LH-20 column was analyzed by gas chromatography without solvolysis, and no peak of bile acid was detected except trace amount of chenodeoxycholic acid. As solvolysis was not carried out in previous works, bile acid sulfate excreted into urine was not determined at all.

Recently glycolithocholic acid sulfate and taurolithocholic acid sulfate were recovered from bile following the oral administration of ^{14}C-lithocholic acid in humans (8,14). The experiments in rat showed that sulfated lithocholic acid was more rapidly excreted in urine than the nonsulfated acid (15).

Further studies of sulfated bile acids in human beings are indicated.

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REFERENCES

1. Rudman, D., and F.E. Kendall, J. Clin. Invest. 36:530 (1957).
2. Makino, I., S. Nakagawa and K. Mashimo, Gastroenterology 56:1033 (1969).
3. Sandberg, D.H., J. Sjövall, K. Sjövall and D.A. Turner, J. Lipid Res. 6:182 (1965).
4. Carey, J.B., Jr., J. Clin. Invest. 37:1494 (1958).
5. Sherlock, S., and V. Walshe, Clin. Sci. 6:223 (1948).
6. Osborn, E.C., I.D.P. Wotton, L.C. Desilva and S. Sherlock, Lancet 2:1049 (1959).
7. Makino, I., S. Nakagawa, K. Shinozaki and K. Mashimo, Lipids 7:750 (1972).
8. Palmer, R.H., Proc. Nat. Acad. Sci. U.S.A. 58:1047 (1967).
9. Norman, A., and B. Strandvik, J. Lab. Clin. Med. 78:181 (1971).
10. Sjövall, J., and R. Vihko, Acta Chem. Scand. 20:1419 (1966).
11. Jänne, O., R. Vihko, J. Sjövall and K. Sjövall, Clin. Chim. Acta 23:405 (1969).
12. Cronholm, T., I. Makino and J. Sjövall, Eur. J. Biochem. 26:251 (1972).
13. Gänshirt, H., F.W. Koss, and K. Morianz, Arzneimittel-Forsch. 10:943 (1960).
14. Palmer, R.H., and M.G. Bolt, J. Lipid Res. 12:671 (1971).
15. Palmer, R.H., Ibid. 12:680 (1971).

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Origin of the High Saturated Fatty Acid Content of Rat Fecal Lipids

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ABSTRACT

The biohydrogenation of unsaturated fatty acids and the preferential absorption of unsaturated fatty acids over long chain saturated fatty acids from the gut have been investigated to find the origin of the high saturated fatty acid content of the fecal lipids of rats fed soybean oil. Label from dietary (1-^{14}C)-linoleic acid was recovered in the saturated and monounsaturated fatty acids of the fecal lipid. However, when (9,10-^{3}H)-stearic acid and (1-^{14}C)-linoleic acid were fed together, the isotope ratio (^{3}H/^{14}C) of the fecal lipid was 1.9 times that of the diet. It is concluded that both processes occur.

INTRODUCTION

The fecal lipids of mammals appear to differ markedly from the dietary lipids both in the lipid class composition and the fatty acid composition (1-7). High concentrations of unesterified fatty acids are found in human (3) and pig (5) fecal lipids, and unpublished observations from our own laboratory suggest the same is true for the rat. The fatty acid spectra show the presence of a number of unusual fatty acids which include branched chain acids (1,2,6,7), hydroxy-substituted fatty acids (1,2), *trans*-unsaturated fatty acids (1,9) and cyclic fatty acids (7). In addition, fecal lipids are characterized by having a very high content of saturated fatty acids (1-7). In the rat fecal lipids, the saturated fatty acids comprise up to 50% of the total fatty acids and those with 15, 16, 18, 24 and 26 carbons make the greatest contributions (7).

Two mechanisms have been proposed to account for the high saturated fatty acid content. These are (a) the hydrogenation of unsaturated fatty acids by the gut microorganisms and (b) the preferential absorption of the unsaturated fatty acids from the gut. The former has been well studied in ruminants (9), and the biochemical sequence for biohydrogenation has been investigated using the rumen micro-organism, *Butyrivibrio fibrisolvens* (10,11). However there is evidence for the selective absorption of unsaturated fatty acids in man (2,4). The rat has bacterial populations between those of ruminants and man (12), and the experiments described in this report were undertaken to find which mechanism(s) was important in the rat.

EXPERIMENTAL PROCEDURES

Adult male albino rats with body weights between 200 and 250 g were fed a base diet (diet B, Ref. 8) supplemented with 10% soybean oil as the sole fat source. The methods of fecal collection and fat extraction and analysis have been described elsewhere (7,8).

The feces were collected from a group of eight rats which had been receiving the soybean oil diet for at least 3 weeks. The animals were killed with carbon dioxide and the total small intestine removed. This was divided into two equal sections (upper and lower) and the lumen contents removed and fat extracted (7).

Two rats were fed 5 g each of the soybean oil diet containing 1.0 μCi (1-^{14}C)-linoleic acid. The unlabeled diet was substituted after 4 hr

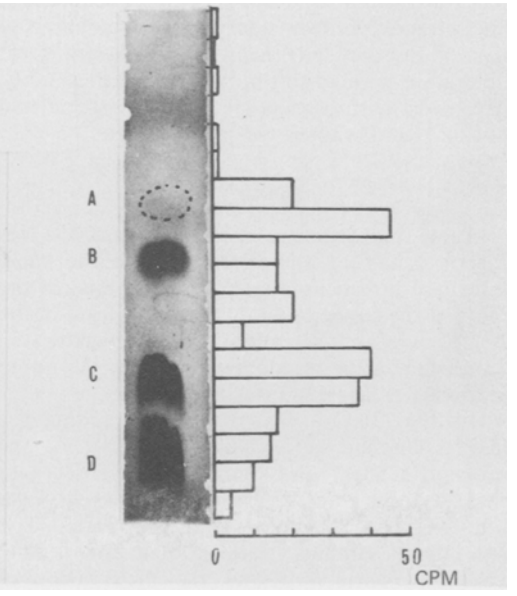

FIG. 1. Distribution of linoleic acid label in rat fecal fatty acids. Plate contains 5% w/w silver nitrate in Silica Gel G. Developed with hexane-diethyl ether 9:1. Charred section is a portion of same layer spotted with a standard mixture of fatty acid methyl esters. (A) 16:0 and 18:0; (B) 18:1; (C) 18:2; and (D) 18:3.

TABLE I

Fatty Acid Composition of Small Intestinal Lumen Contents and Fecal Lipids of Rats Fed Soybean Oil[a]

Component	18:0	18:1	18:2	18:3
Soybean oil	5.5	23.6	61.8	10.1
Small intestine lumen[b]				
Upper	17.4 (3.3)	17.9 (1.8)	59.5 (2.5)	5.1 (1.3)
Lower	20.7 (3.7)[c]	28.1 (4.4)[c]	48.3 (4.5)[c]	2.9 (0.9)
Feces[d]	45.1 (8.4)[c]	34.0 (7.7)[c]	20.9 (10.3)[c]	---

[a]Percentage of total 18-carbon acids.

[b]Mean and standard deviation of eight estimations.

[c]$P<0.001$ values compared with upper lumen contents.

[d]Five estimations; 18-carbon iso-branched acid not included.

and the feces collected over the next 4 days. Samples of the fecal lipid with the greatest specific activity were saponified and methyl esters formed from the liberated fatty acids. These were applied to silver nitrate impregnated thin layers of Silica Gel G (5% w/w), which were developed with hexane-diethyl ether 9:1. Part of the layer was removed in 1 cm strips and counted for radioactivity, and the remainder was sprayed with an acid solution of ammonium sulphate and charred (14).

Finally, four rats were fed 5 g each of the soybean oil diet containing 8.0 μCi (9,10-^{3}H)-stearic acid and 2.2 μCi (1-^{14}C)-linoleic acid, following which the unlabeled diet was substituted. The feces were collected, the fat extracted and the isotope ratio compared to that of the diet fed. Samples of the total fecal lipid were applied to thin layers of Silica Gel G, developed with hexane-diethyl ether-acetic acid 80:20:1, and assayed for radioactivity.

RESULTS

Table I shows the composition of the 18-carbon acids of the dietary lipid, the small intestinal lumen lipid and the fecal lipid of the rats fed the soybean oil diet. The increase in the stearic acid content relative to the unsaturated 18 carbon acids is apparent even in the upper sections of the small intestine.

In the first 4 days following feeding the (1-^{14}C)-linoleic acid, 0.8% and 1.3% of the ingested activity was excreted in the feces of the two rats studied. The greatest activity was found in the feces passed in the second day, and these samples were pooled for further analysis. Figure 1 shows the distribution of activity among the different classes of fatty acid methyl esters. While greatest activity (43% of the recovered activity) was found in the zone containing the diunsaturated esters, 28% and 14% of the total ester activity was recovered in the zones containing the saturated and monounsaturated esters, respectively.

The mean ^{3}H/^{14}C ratio of the fecal lipid of the rats fed the double-labeled diet was 6.7 (range 5.8-8.6). This is 1.9 times the isotope ratio of the diet fed. For each animal the total recovery of activity of either isotope during the first 4 days was less than 5% of that ingested. Of the total fecal lipid, 61% of the activity was located in the free fatty acid fraction.

DISCUSSION

In previous experiments we have shown that rats treated with neomycin excrete much less saturated lipid compared to untreated controls (7). The excretion of both saturated fatty acids and saturated sterols decreased during treatment, while that of the unsaturated fatty acids and unsaturated sterols increased. Bottino (6) has reported similar observations for rats fed methyl esters of unsaturated fatty acids and treated with tetracycline-neomycin-nystatin mixture. Both these observations are consistent with bacterial hydrogenation of the unsaturated fatty acids occurring within the lumen.

Our observations concerning the changes in the 18-carbon acids described here could result from either of the two mechanisms under study. While rodents have high concentrations of bacteria in the small intestine (12), most of fatty acid absorption occurs in the jejunum (13) in the upper portion of the small intestine. That biohydrogenation does occur is shown in the recovery of the activity from the dietary (1-^{14}C)-linoleic acid in the saturated and monounsaturated fatty acids of the fecal lipid.

However the results from the double labeled experiment show that there is, as well, a preferential absorption of the unsaturated fatty acids at the expense of the long chain saturated fatty acids from rat small intestine. Thus both mechanisms appear to be responsible in pro-

ducing the high saturated fatty acid content of the fecal lipids of rats fed soybean oil.

REFERENCES

1. James, A.T., J.P.W. Webb and T.D. Kellock, Biochem. J. 78:333 (1961).
2. Gompertz, S.M., and H.G. Sammons, Clin. Chim. Acta 8:591 (1963).
3. Williams, J., A. Sherma, L.J. Morris and R.T. Holman, Proc. Soc. Exp. Biol. Med. 105:102 (1960).
4. Fernandes, J., J.H. van der Kamer and H.A. Weijers, J. Clin. Invest. 41:488 (1962).
5. Hamilton, R.M.G., and B.E. MacDonald, J. Nutr. 97:33 (1969).
6. Bottino, N.R., Lipids 2:155 (1967).
7. Grigor, M.R., G.G. Dunckley and H.D. Purves, Biochim. Biophys. Acta 218:400 (1970).
8. Grigor, M.R., G.G. Dunckley and H.D. Purves, Ibid. 218:389 (1970).
9. Garton, G.A., World Rev. Nutr. Dietetics 7:225 (1967).
10. Kepler, C.R., W.P. Tucker and S.B. Tove, J. Biol. Chem. 246:2765 (1971).
11. Rosenfeld, I.S., and S.B. Tove, Ibid. 246:5025 (1971).
12. Savage, S.C., R. Dubos and R.W. Schaedler, J. Exp. Med. 127:67 (1968).
13. Johnston, J.M., Proc. Soc. Exp. Biol. Med. 100:669 (1959).
14. Ziminski, T., and P.E. Borowski, J. Chromatogr. 23:480 (1966).

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Brain Cholesterol: XVI. Incorporation of Different Precursors into Baboon Tissue Sterol

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ABSTRACT

The relative order of incorporation of 1-C^{14}-acetate and 2-^{3}H-acetate into tissue cholesterol was similar. Short term fasting tended to decrease incorporation of acetate into liver and adrenal sterol. Other tissues, particularly neural, had the same or showed an increased incorporation. This relative order of incorporation found for acetate was different when U-^{14}C-glucose was used. Cholesterol values from neural tissue after glucose were greater than non-neural sterol values.

INTRODUCTION

Every mammalian tissue so far tested has been shown to be capable of cholesterogenesis (1-3). Because early experimental evidence suggested that liver was the major source of circulating sterol (4-7), primary interest in the field centered around hepatic sterol metabolism. For some time it was felt that physiological control of sterol balance was reflected primarily by the rate of hepatic steroidogenesis (4,8-10). Thus, while fasting and the cholesterol content of the diet have both been shown to markedly influence cholesterogenesis by the liver, only a few studies have appeared in which control mechanisms have been evaluated in extrahepatic tissues (3,10-14). Even less is known concerning these same factors on brain sterol metabolism. Although adult brain lipid levels have been reported as not being affected by starvation (15), results of Kabara (16) in mice and Smith's in vitro experiments with rat brain (17) suggested that cerebral sterol metabolism could be altered but in a manner different from that of hepatic biosynthesis.

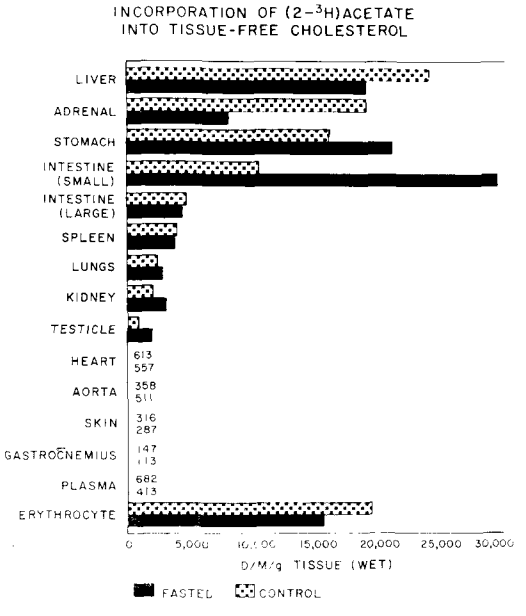

FIG. 1. Values obtained from baboon tissue 1 hr after intravenous injection of precursor. Fasted group received no food for 18-20 hr.

Little is known of the systems that govern the metabolism of sterols in brain (18,19). Most studies dealing with brain cholesterol metabolism have been limited to the use of whole brain rather than regional areas. Studies dealing with small animals usually exclude the possibility of such regional samples for study. The present experiments were carried out on a nonhuman primate in order to rectify this situation and also to develop a data bank on brain cholesterol metabolism which would be useful in understanding brain metabolism in primates. Although emphasis has been placed on our brain data, results of precursor incorporation into other tissue are also presented. These data for other tissues may be useful information to our understanding of cholesterol metabolism in general and as contrasted with neural sterol metabolism in particular.

The primary objective of the present experiments was to measure the effect of a short period of food removal (18 hr) on brain and extraneural tissue sterol metabolism. Rates of metabolism were determined by use of acetate-1-C^{14}, acetate-2-H^{3} and glu-U-C^{14} as precursors to tissue cholesterol. Therefore these experiments provide information on (a) relative rate of cholesterogenesis in the regional areas of the baboon brain, (b) pattern of labeling with different precursors, (c) pattern of incorporation of the same precursor labeled in different positions and (d) effect or lack of effect of short fasting upon the rate of cholesterogenesis in different tissues as measured by 2-^{3}H acetate incorporation.

EXPERIMENTAL PROCEDURES

Animal Preparation

Five male zoo baboons and one African

baboon, weighing between 10 and 12 kg representing ages ca. 2-4 years old, were used for these studies. The African animal was imported from Kenya, East Africa. All animals were colonized for ca. 1-2 years before use. Diet during captivity consisted of monkey chow (Ralston Purina Co., St. Louis, Mo.) supplemented with fresh fruit and whole corn. All animals were clinically healthy and for the purpose of these experiments, divided into two groups. The first group (control) was fed on the usual diet while a second group was fasted for ca. 18-20 hr before isotope injection.

All animals were anesthetized in these experiments with phencyclidine (Sernylan, 2 mg/kg). The unconscious animals were injected intravenously with 10 μC/kg of 1-^{14}C-acetate (7.0 mC/mM) and 100 μC/kg of 2-^{3}H-acetate (100 mC/mM) (Basal Control); the second group of animals was injected with 10 μC/kg of glucose-U-C^{14} (6.66 mC/mM) and 100 μC/kg of 2-^{3}H-acetate (44 mC/mM) (fasted group).

Blood samples were taken at 30 and 60 min. Animals were killed at the end of 1 hr by injection of air and autopsied within the hour. Samples of various tissues were removed, washed in cold distilled water, blotted dry and quick frozen in a dry ice-acetone mixture, and stored in frozen state (-70 C).

Free and esterified (after hydrolysis) sterols were isolated, measured and assayed by the method previously reported (20). Radiochemical purity of the isolated free cholesterol was established (20,21). The great majority of sterol radioactivity (>85%) represents cholesterol in all tissues except skin (2,22). Hence sterol radioactivity represents primarily labeled cholesterol and precursor incorporation rates are a *relative indicator* of cholesterol biosynthesis.

Data were calculated on the basis of activity per gram wet tissue for each isotope. Since a number of important variables (pool size, tracer concentration at site of synthesis, etc.) could not be measured, the data were not present in terms of micromoles of precursor incorporated. While mathematically it was possible to relate D/M/mg into micromoles, the resulting numbers give the results a degree of "sophistication" not warranted by the data. Also the generalizations made from the data did not depend upon, nor would they be influenced by, such a presentation.

RESULTS

In separate experiments three animals were injected with 2-^{3}-acetate and 1-C^{14} acetate while a second set of three baboons was fasted prior to injection with 2-^{3}H-acetate and

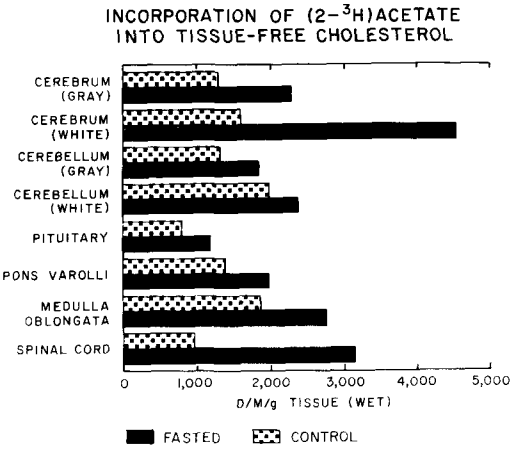

FIG. 2. Neural cholesterol values obtained from same animals as Figure 1.

U-C^{14}-glucose. In order to evaluate the effect of short term fasting, the first group of animals (control) was compared to a group of baboons fasted for 18 hr. Mean rates of incorporation are presented as bar lengths on the graph. Blood, 13 organs (Fig. 1) and eight tissue samples of the central nervous system (Fig. 2) were examined.

Incorporation of 2-^{3}H-acetate into hepatic sterol was suppressed by short term fasting, but not as markedly as in the adrenal gland. In the gastrointestinal tract of the baboon, fasting resulted in some increase of acetate incorporation, especially in the small intestine (ileum). All other tissue, with the possible exception of a slight increase in kidney and testicle values, showed no significant changes.

Tissue samples from the brain and spinal cord were removed surgically and represented discrete but not pure anatomical areas. Sterol isolated from these tissues indicated that short term fasting increases rather than decreases the amount of 2-^{3}H-acetate incorporated into neural sterols. Values for cholesterol isolated from the spinal cord indicated the largest relative increase.

Incorporation of 1-C^{14} Acetate into Baboon Sterols

The relative rates of tissue biosynthesis as measured by 1-C^{14}-acetate incorporations are similar to those found for 2-^{3}H-acetate (Table I). Again liver carbon-14 values were higher than any other tissue examined. The order of incorporation in the various tissues was remarkably similar for both acetate precursors. Differences between white and gray matter values were less pronounced in these series (Table I). than in the first group (Fig. 2). White matter

TABLE I

Precursor Incorporation into Tissue-Free Cholesterol

Organ	mg/g Wet tissue	D/mg/g Wet tissue	
		(1-^{14}C) Acetate	(U-^{14}C) Glucose
Liver	1.50 ± 0.66	68,286 ± 15,107	409 ± 111
Adrenal	2.00 ± 0.87	38,309 ± 3,037	237 ± 113
Stomach	1.68 ± 0.22	27,056 ± 9,505	525 ± 96
Intestine (small)	1.70 ± 0.26	23,214 ± 5,574	448 ± 102
Intestine (large)	1.30 ± 0.54	8,938 ± 2,175	468 ± 46
Spleen	2.47 ± 0.80	8,193 ± 1,273	316 ± 89
Lungs	1.76 ± 1.10	4,496 ± 932	344 ± 90
Kidney	2.77 ± 0.46	3,593 ± 1,191	916 ± 180
Testicle	1.05 ± 0.48	1,086 ± 267	510 ± 222
Heart	0.86 ± 0.61	1,024 ± 321	224 ± 103
Aorta	0.56 ± 0.47	510 ± 306	440 ± 191
Skin	0.15 ± 0.18	599 ± 335	138 ± 52
Gastrocnemius	0.09 ± 0.06	118 ± 34	33 ± 1
Plasma	0.04 ± 0.01	767 ± 715	25 ± 16
Erythrocyte	1.73 ± 0.53	8,113 ± 6,605	194 ± 106
Cerebrum			
Gray	12.6 ± 2.6	1,056 ± 136	3,680 ± 605
White	26.3 ± 8.1	1,168 ± 366	3,603 ± 905
Cerebellum			
Gray	10.2 ± 1.4	749 ± 183	2,947 ± 844
White	22.7 ± 6.3	641 ± 100	4,446 ± 1,063
Pituitary	0.73 ± 0.94	1,104 ± 51	155 ± 110
Pons varolli	21.3 ± 7.5	1,135 ± 195	2,573 ± 795
Medulla oblongata	18.7 ± 9.5	710 ± 69	1,368 ± 321
Spinal cord	15.9 ± 5.3	736 ± 145	2,114 ± 276

had approximately the same amount of radioactivity as gray matter in the first hour after precursor injection.

Incorporations of Glucose-U-C^{14} into Baboon Sterols

The relative metabolic pattern exhibited by glucose incorporation into the sterol of various tissues was completely different from that given by acetate incorporation. Consequently one can only discuss the relative incorporation of a precursor, rather than emphasizing biosynthetic rate. Of interest is the high incorporation of U-C^{14} glucose into kidney and testicular tissue (Table I). The gastrointestinal specimens yielded high sterol activity as contrasted with cholesterol isolated from liver and adrenal, the two tissues showing high incorporation of acetate. Again, as with the acetate incorporation values, skin and muscle values are low.

In sharp contrast to values obtained for brain cholesterol after acetate incorporation, the radioactivity isolated from regional areas of the brain after glucose U-C^{14} is high as compared to other organs. Also the comparison of neural sterol counts with that of hepatic and other extrahepatic tissue after this precursor indicates a relatively high amount of metabolic activity for brain. While cerebrum gray and white area sample values were similar, cerebellar white area samples contained relatively more radioactivity (D/m/g wet tissue) than did sterol isolated from cerebellar gray areas. All other neural samples, except for pituitary values, were higher than non-neural tissue values after U-^{14}C-glucose as compared to 1-^{14}C-acetate.

DISCUSSION

Virtually all investigations on the elucidation of mechanisms of cholesterogenesis in various organs have been carried out in lower animals (12,23-25). Several observations in primates (3,22,26-28) suggest that differences exist in hepatic and extrahepatic synthetic capacity. However only three comparative observations are available concerning cholesterol metabolism in brain of primates (3,29,30). Therefore the present investigation was undertaken to further investigate regional biosynthesis of brain cholesterol in the baboon and to compare the effect of short term fasting on this process.

These experiments give a comparison of the relative rates of precursor incorporation per unit wet weight of various tissues. Because various precursor pools in each tissue are unknown quantities, statements concerning the overall rate of sterol synthesis per tissue or whole organ cannot be made with any degree of certainty. Attempts to calculate the data to such a basis would give values and interpretation not justified by experimental results.

The present report represents data from six baboons divided into two groups. In such a limited series, individual variation becomes an important factor. Despite these limitations, some interesting conclusions can be made. The data supports previous general conclusions for tissue cholesterogenesis (3,29) and thus those parameters, acetate vs. glucose incorporation and regional brain biosynthesis, which are unique to the present experiments, can be accepted with some degree of confidence.

Fasting and the cholesterol content of the diet have both been shown to influence cholesterogenesis (2,3 and reference therein). While the differences for any one organ prevent one from making strong conclusions, there is a general trend. This trend suggests that sterol synthesis in extrahepatic tissue after fasting is not decreased to the same extent as in liver, and supports the general conclusions reached by Dietsch and coworkers (2,3). Also incorporation values for cholesterol isolated from brain are higher after fasting rather than lower. This agrees with our previous results with acetate and glucose in mice (16). Because it has been shown that starvation produces ketosis in primates (31), that ketone bodies (aceto acetate) are readily taken up by the brain (32 and references therein) and that ketone bodies partially replace glucose as fuel (33), the interpretation for the changes in incorporation between acetate and glucose during food restriction is difficult. Consequently the increase noted for acetate incorporation into brain sterol during fasting may be a reflection of a number of competing reactions: less incorporation into non-neural tissue would make more precursor available to the brain; increase utilization of noncarbohydrate precursor by the increase in precursor pool due to fasting. The present data does not differentiate the contribution of these variables to the increase of acetate incorporation.

Regardless of the mechanism involved, our in vivo results indicate relative rates of precursor incorporation into sterol of various baboon tissue different from those found in vitro for tissue values from the monkey (3). Whether the difference is a reflection of species or technique, difference must be considered. Since in vitro incubation may not reflect true organ capabilities especially for brain (34), the present in vivo studies may give a truer picture of rates of cholesterogenesis.

Since the rate of organ synthesis as measured by precursor incorporation is a function of diet, precursor used, and general health and condition of the animal being studied, the use of fasting animals and specific isotopes changed the relative ratio of precursor incorporation between tissues. Our data on liver, small intestine, stomach and adrenal gland show high incorporation of acetate-2-H^3, while regional areas of the brain have cholesterol specific activity of intermediate value. This same generalization holds for acetate-1-C^{14}. In contrast, however, the relative tissue sterol incorporation values after Glu-U-C^{14} are markedly different. With this precursor and excluding brain values, there is less distinction between incorporation values for various tissues obtained with glucose as compared to acetate. Of further significance are the higher values for all regional areas of the brain, except pituitary, as compared to non-neural tissue. Therefore these experiments in the baboon substantiate earlier findings which contrasted the incorporation rate of acetate and glucose into neural and non-neural tissue of the mouse (35). Thus brain biosynthesis as measured by acetate (fatty acid) incorporation does not necessarily reflect the true synthetic capacity of this or any other tissue. Although both precursors are taken up rapidly by the brain from circulating blood (36 and references therein), it remains to be determined just what foodstuffs are used by neural tissue for sterol synthesis during development and growth.

The present sterol data from gray and white matter of the cerebral and cerebellum area do not support our previous conclusions (29) or those of others (37,18). The main difference seems to be the short duration between isotope injection and killing of the animal. Because of this, the present protocol may be closer to revealing true in vivo conditions than previous studies. Results with acetate-2-H^3 indicate that after 1 hr there is a greater incorporation of isotope into cerebral and cerebellum sterol of white than gray matter. With acetate-1-C^{14}, no such differences were noted. Using Glu-U-C^{14} no difference between cereb ral white and gray matter was measured, while sterol isolated from cerebellum white matter was higher in activity than that isolated from cerebellum gray matter.

While the above values are of interest, they suffer from the fact that the tissue samples designated white and gray matter really mean enriched areas rather than single anatomical entities, since surgical dissections were made and there is doubt as to the homogeneity of the samples examined. Methodology available at the time these pilot experiments were carried out did not permit a more elegant approach to the problem.

Values obtained for sterols isolated from the pituitary after administration of glucose are of special interest. In contrast to all other regional brain values, the radioactivity for pituitary

cholesterol is ca. 10-fold lower than other brain values and is comparable to non-neural tissue. The endocrine nature of this structure and its embryonic history may account for our findings. Whether this represents an explanation or not will depend on other critical experiments focusing on this general problem of lipid biosynthesis in the diencephalon area of the brain.

In this respect the data from other neural tissue (pons varolli, medulla oblongata and spinal cord) indicate that greater differences between these individual neural areas are recorded after acetate-1-C^{14} than acetate-2-H^3 or even glucose-U-C^{14}. However, since tissue specimens were not taken for histological examination, too much emphasis concerning regional area differences in our preliminary findings may be misleading.

Future research will deal with the in vivo uptake of precursor into different anatomical areas of the brain and the isolation of neuronal- and glial-enriched cell populations. Nicholas and coworkers (38) have recently reported on in vitro lipid biosynthesis in neuronal and glial-enriched fractions of rat brain. Using 2-^{14}C mevalonic acid, the neuron-enriched fraction (gray matter) formed much less neutral lipid than did glial-enriched (white matter). Their preliminary and unpublished observation of results after intraperitoneal injection of 2-^{14}C-acetate (8 hr prior to isolation) indicated that 97% of the labeled isoprenoid material isolated from the two cell types was found in the glial-enriched fraction. However, because 8 hr represents a point well past the peak of precursor incorporation, a more complete time vs. specific activity plot, especially at early time intervals, needs to be carried out. Future experiments in our laboratory will provide in vivo data for early regional biosynthesis in young and adult animals using the fractionation technique of Nicholas et al. (38) and Sellinger et al. (39).

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REFERENCES

1. Srere, P.A., I.L. Chaikoff, S.S. Treitman and L.S. Burstein, J. Biol. Chem. 182:629 (1950).
2. Dietschy, J.M., and M.D. Siperstein, J. Lipid Res. 8:97 (1967).
3. Dietschy, J.M., and J.D. Wilson, J. Clin. Invest. 47:166 (1968).
4. Gould, R.G., Amer. J. Med. 11:209 (1951).
5. Friedman, M., S.O. Byers and F. Michaelis, Amer. J. Physiol. 164:789 (1951).
6. Harper, P.V., W.B. Neal, Jr., and G.R. Hlavacek, Metabolism 2:69 (1953).
7. Hotta, S., and I.L. Chaikoff, Arch. Biochem. Biophys. 56:28 (1955).
8. Tomkins, G.M., H. Sheppard and I.L. Chaikoff, J. Biol. Chem. 201:137 (1953).
9. Langdon, R.G., and K. Bloch, Ibid. 202:77 (1953).
10. Tomkins, G.M., and I.L. Chaikoff, Ibid. 196:569 (1952).
11. Siperstein, M.D., and M.J. Guest, J. Clin. Invest. 39:642 (1960).
12. Gould, R.G., C.B. Taylor, J.S. Hagerman, I. Warner and D.J. Campbell, J. Biol. Chem. 201:519.
13. Cox, G.E., L.G. Nelson, W.B. Wood and C.B. Taylor, Fed. Proc. 13:31 (1954).
14. Dietschy, J.M., and M.D. Siperstein, J. Clin. Invest. 44:1311 (1965).
15. Folch, J., "Psychiatric Research," Drinker, Howard University Press, Cambridge, Mass., 1947, p.23.
16. Kabara, J.J., "VII International Congress of Neurology," 1961, p. 725.
17. Smith, M.E., J. Neurochem. 10:531 (1963).
18. Korey, S.R., and A. Stein, in "Regional Neurochemistry Proceedings," International Neurochemistry Symposiums, Varenna, Italy, Edited by S.S. Kety and J. Elkes, Pergamon Press, New York, 1967, p. 175.
19. Kabara, J.J., in "Advances in Lipid Research," Vol. 5, Edited by R. Paoletti and D. Kritchevsky, Academic Press, New York, 1967, p. 279.
20. Kabara, J.J., J.T. McLaughlin and C.A. Riegel, Anal. Chem. 33:305 (1962).
21. Kabara, J.J., and J.T. McLaughlin, J. Lipid Res. 2:283 (1961).
22. Wilson, J.D., J. Clin. Invest. 47:175 (1968).
23. Kabara, J.J., JAOCS 42:1003 (1965).
24. Schwenk, E., G.J. Alexander and C.A. Fish, Arch. Biochem. Biophys. 58:37 (1955).
25. Jansen, S.R., M.E. Zanetti and C.F. Hutchison, Ibid. 138:433 (1970).
26. Taylor, C.B., D. Patton, N. Yogi and G.E. Cos, Proc. Soc. Exp. Biol. Med. 103:768 (1960).
27. Kapan, J.A., G.E. Cox and C.B. Taylor. Arch. Pathol. 76:359 (1963).
28. Mann, G.V., Circ. Res. 18:205 (1966).
29. Kabara, J.J., and N.T. Werthessen, Lipids 2:494 (1967).
30. Plotz, E.J., J.J. Kabara, M.E. Davis, G.V. LeRoy and E.G. Gould, Amer. J. Ob. and Gyn. 101:534 (1968).
31. Friedemann, T.E., J. Biol. Chem. 142:635 (1942).
32. Persson, B., G. Settergren and G. Dahlquist, Acta Paediat. Scand. 61:273 (1972).
33. Balasse, E.O., Herm. Metabol. Res. 3:403 (1971).
34. Paoletti, E.G., "Advances in Experimental Medicine and Biology", Vol. 13, Plenum Press, New York, 1971, p. 41.
35. Kabara, J.J., Progress in Brain Res. 9:155 (1964).
36. Dhopeshwarkar, G.A., C. Subramanian, D.M. McConnell and J.A. Mead, Biochim. Biophys. Acta. 255:572 (1972).
37. Davison, A.M., J. Dobbing, K.S. Morgan and G.P. Wright, Lancet 1:658 (1959).
38. Jones, J.P., R.B. Ramsey and H.J. Nicholas, Life Sci. 10:997 (1971).
39. Sellinger, O.Z., J.M. Azcurra, D.E. Johnson, W.G. O'Hesson and Z. Lodin, Nature New Biol. 230:253 (1971).

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Influence of the Polar Group on Interactions between Phospholipids and Sterols in Monolayers. Rapid Decomposition of Phosphatidylethanolamines and Cholesterol in Monolayers at pH 11.9

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ABSTRACT

Pressure-area curves were obtained for monolayers of pure lipids or mixtures of lipids in order to investigate the effect of different polar groups upon interactions between molecules of various structures. Cholest-4-ene-3-one and cholestan-3-one were measured alone and in mixtures with di 16:0-lecithin, 16:0-18:1-lecithin, 16:0-18:3-lecithin, and 16:0-20:4-lecithin. All lecithin films were somewhat condensed by the ketones, but much less so than by cholesterol. Cholesterol was measured alone and with either di 16:0 phosphatidylethanolamine (PE) or rat liver phosphatidylethanolamine at pH 2.0, 5.4 and 11.9. Cholesterol condensed the natural PE at pH 2.0 and 5.4, but not the di 16:0 PE. At pH 11.9, both cholesterol and PE were chemically unstable in monolayers. The strong interaction of cholesterol with mixed phosphatidylethanolamines in monolayers suggests that cholesterol may also interact strongly with PE in membranes, of which PE are important components.

INTRODUCTION

There has been much work on the interactions between lecithins and sterols, particularly cholesterol, in monolayers, and in most of these studies the polar group interactions were between the glycerylphosphorylcholine moiety and the 3-β hydroxyl group (1-9). Attention was mainly focused upon the interactions between the hydrocarbon parts of the molecules. The next logical step would be to learn how different polar groups affect intermolecular interactions in monolayers, with the hydrocarbon part of the molecule remaining constant. Therefore we decided to investigate how the substitution of a keto group for a hydroxyl group would affect sterol behavior, so cholestan-3-one and cholest-4-ene-3-one were examined for comparison with cholestanol and cholesterol. Demel et al. (5) have studied these ketones (among others) in combination with oleoyl-stearoyl-lecithin (18:1-18:0), a lecithin which is rare or nonexistent in nature. It was therefore useful to study these ketones in combination with lecithins of structure that are known to occur in membranes, and also to compare their behavior with that of cholesterol or cholestanol in combination with the same lecithins, in order to distinguish between the effects of the polar groups and those of the hydrocarbon moieties.

We also wanted to compare the behavior of the unsubstituted amino group of phosphatidylethanolamine (PE) with that of the trimethylamino group in lecithin. An attempt to investigate the interaction of the uncharged amino group with cholesterol on a pH 11.9 subphase failed, because monolayers of both cholesterol and PE were unstable under these conditions (see below). Also, we hoped to learn whether highly unsaturated PE (which are abundant in certain membranes) would condense with cholesterol, since previous work (1,2) on individual PE species including 16:0-16:0, 18:0-18:1, 16:0-18:2, 16:0-18:3 and 18:3-16:0 had indicated that only 18:0-18:1 would condense in a monolayer with cholesterol.

EXPERIMENTAL PROCEDURES

Cholesterol (Nutritional Biochemicals Corp.,

TABLE I

Fatty Acid Composition of Rat Liver Phosphatidylethanolamine

Fatty acid	Mol %
16:0	18.0
18:0	35.5
18:1	5.1
18:2	7.8
18:3	1.6
20:2[a]	1.2
20:3[a]	0.8
20:4	20.7
22:4[a]	1.5
22:5[a,b]	3.0
22:6	4.6

[a]Tentatively identified by relative retenion time.
[b]Two components, probably isomers.

TABLE II

Decrease in Area Per Molecule Produced by Mixing of Lecithins with Cholest-4-ene-3-one or Cholestan-3-one in Monolayers ($Å^2$)

		Mole Fraction of Lecithin					
		$Å^2$	$\Delta Å^2$				$Å^2$
Lecithin	Sterol	0	0.2	0.4	0.6	0.8	1.0
				5 dynes/cm			
16:0-16:0	Cholest-4-ene-3-one	55.3	5.5	9.4	11.5	4.5	92.5
16:0-18:1			6.5	9.6	7.8	4.1	101.5
16:0-18:3			6.0	12.0	12.1	6.0	116.3
16:0-20:4			3.7	5.3	4.6	3.5	111.0
16:0-18:1	Cholestan-3-one	44.2	5.1	9.0		0.9	101.5
16:0-20:4			10.2	8.2		5.4	111.0
				20 dynes/cm			
16:0-16:0	Cholest-4-ene-3-one	47.2	0.2	1.4	2.0	2.2	62.0
16:0-18:1			4.4	8.0	6.2	3.8	79.2
16:0-18:3			3.3	7.9	10.6	6.0	88.6
16:0-20:4			6.3	8.2	8.0	4.6	84.8
16:0-18:1	Cholestan-3-one	40.6	3.6	9.5		1.8	79.2
16:0-20:4			7.8	7.0		4.9	84.8

Cleveland, Ohio) was checked for purity by thin layer chromatography (TLC) (10,11) and only one component was found. Cholest-4-ene-3-one (Eastman Organic Chemicals, Rochester, N.Y.) was recrystallized from absolute ethanol. It melted at 80 C (lit. 82 C [12]) and gave only one spot on TLC (10). Cholestan-3-one (Aldrich Chemical Co., Milwaukee, Wis.) melted at 127-8 C (lit 129 C [12]) and produced only one spot on TLC (10). These lipids were dried over P_2O_5 under vacuum for 12 hr or more at room temperature, and a weighed amount of each was dissolved in redistilled reagent grade benzene.

Dipalmitoyl lecithin (Nutritional Biochemicals Corp.) and dipalmitoyl PE (Calbiochem, La Jolla, Calif.) were analyzed by TLC (13) and each produced only one spot. Gas chromatographic (GLC) analyses of these indicated that palmitic acid accounted for more than 99% of the fatty acids in each case. The preparation and purification methods for 1-palmitoyl-2-oleoyl-lecithin (16:0-18:1), 1-palmitoyl-2-linolenoyl-lecithin (16:0-18:3) and 1-palmitoyl-2-arachidonoyl-lecithin (16:0-20:4) have been described earlier (7). These compounds were at least 97% pure, as found by GLC analysis of the fatty acid mole ratio. Natural PE was a mixture of many molecular species isolated from rat liver lipid by preparative TLC (13). Its fatty acid composition is given in Table I.

Solutions of a sterol in benzene, or a phospholipid in a mixture of chloroform-methanol (dipalmitoyl PE was dissolved in chloroform-ethanol) were mixed before spreading of films.

Pressure-area measurements were made at 22 ± 1 C with a Cenco Hydrophil Balance (Central Scientific Co., Chicago, Ill.) as described before (4). The subphase was distilled water (measured pH ca. 5.4), 0.01 M HCl (measured pH 2.0) or 0.01 M KOH (measured pH 11.9). Lipid solutions were measured onto the aqueous phase with a Hamilton syringe (Hamilton Co., Whittier, Calif.), and at the same time an equal aliquot was taken for GLC analysis of fatty acid content to confirm the concentration of phospholipid (14) (all solutions except pure sterols). Each solution was spread and analyzed at least twice. A complete set of mixtures and pure components was measured on 1 day to minimize variations caused by slight temperature changes, etc. Maximum variation in area obtained for the same sample measured on different days was ± 2Å, but usually was much less.

RESULTS AND DISCUSSION

Cholest-3-one and cholest-4-ene-3-one produced films with considerably greater areas per molecule than cholestanol or cholesterol (not shown), as has been reported before (5,8). These films could not be compressed beyond ca. 20 and 28 dynes/cm, respectively, which suggests that the keto group interacts much more weakly than the hydroxyl group with the aqueous phase. In monolayers of cholesterol, the rigid steroid ring system is oriented vertically (15), but in monolayers of these ketones the molecules apparently are tilted so that they occupy larger areas than cholesterol does. Cholest-4-ene-3-one appears to be more tilted than cholestan-3-one (larger area per molecule).

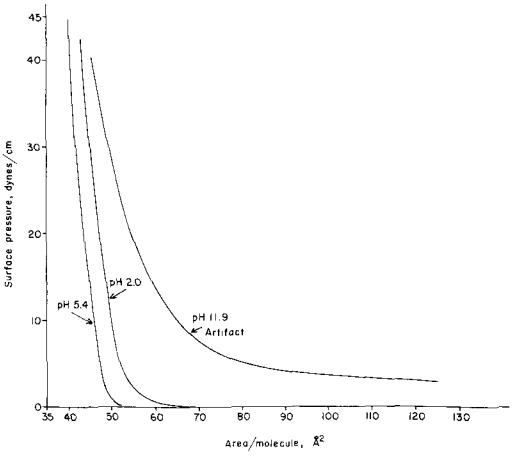

FIG. 1. Effects on the pressure-area curves of dipalmitoyl PE of subphases of pH 2.0, 5.4 and 11.9.

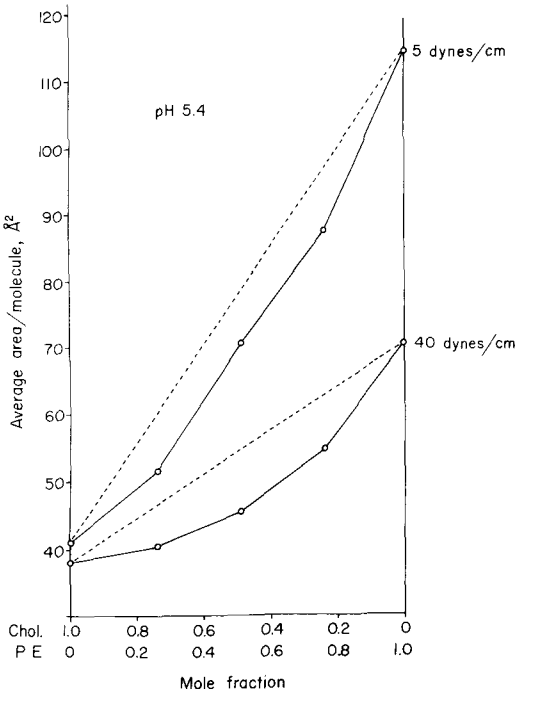

FIG. 2. Interaction of rat liver PE with cholesterol in monolayers on subphase pH 5.4 at 22 ± 1 C.

Both ketones produced condensations with each lecithin, and the reductions in area per molecule in the mixed monolayers were similar for both ketones (Table II). This condensation was smaller than that produced by cholesterol with these lecithins and is about the same as the condensation produced by cholestanol (8). That is, cholestanol and the two ketones are roughly equivalent in condensing these lecithins, and cholesterol is much more effective than they are.

The double bond at carbon 4 of cholesterol causes it to be a stronger condensing agent than cholestanol, but the double bond in cholest-4-ene-3-one did not produce condensations greater than those observed with cholestan-3-one. Therefore it is the combination of the 3-β-hydroxyl and the double bond at carbon 4 that is most effective in producing condensations in mono-layers of these lecithins.

It is interesting that Demel et al. (5) found that these ketones can also condense monolayers of an "inverted" lecithin (18:1-18:0) and, similarly, that the effect was smaller than that of cholesterol with this lecithin. These results imply that the relative positions of the saturated and unsaturated chains in the lecithin molecule are not important in the condensation of lecithins by these ketones.

Synthetic (16:0-16:0) PE, natural PE and cholesterol were measured, separately and in mixtures at pH 2.0, 5.4 and 11.9, in an effort to study the effect of the ionization of the amino group on cholesterol-PE interactions. At pH 11.9, cholesterol produced films with surprisingly large areas per molecule, which bore a suspicious resemblance to the data obtained by Kamel et al. when cholesterol monolayers were allowed to oxidize in air for an hour on a subphase of pH 7.0 (16). We therefore spread and collected films of cholesterol at each pH and tested these materials, along with the stock cholesterol solution, by TLC (one dimension [16]). Five new components were found in the sample exposed to pH 11.9, but the others all appeared to be pure. A similar experiment with 16:0-16:0 PE films and stock solution showed that the PE had completely disappeared, and that three new components had appeared in the sample collected at pH 11.9 (TLC [13]). The unspread sample and those spread at pH 2.0 or 5.4 seemed unaffected. These observations show that monolayers of cholesterol or PE can decompose very rapidly when exposed to a subphase of pH 11.9. These phenomena may be very important in the interpretation of certain data in the literature. For example, in a report concerning the behavior of 16:0-16:0 PE monolayers at various pH values, at pH 11.9 the authors obtained a pressure-area curve very similar to ours (Fig. 1). Our results suggest that those experiments, also, probably involved decomposition of PE, and that the physical-chemical interpretation made by these authors should be reevaluated (17). Such rapid decomposition of both cholesterol and PE suggests that these molecules also may be unstable at

high pH in other highly dispersed preparations, such as multibilayers. A recent investigation of pH effects on the physical behavior of multibilayers of PE or PE plus cholesterol indicated that there was a sudden change in the properties of the preparations between pH 8.3 and 10.4 (18). Before the interpretation of these results can be fully accepted, it should be shown that the lipid had not decomposed sufficiently to produce important changes in the physical properties of the multibilayer preparations.

Cholesterol and the PE samples were stable at pH 2.0 and 5.4. Dipalmitoyl PE did not condense with cholesterol, as had been found by other investigators, but the natural PE was condensed considerably both at pH 2.0 (not shown) and at pH 5.4 (Fig. 2). Other workers have shown that 18:0-18:1 PE will condense with cholesterol, but that 16:0-16:0, 16:0-18:2, 16:0-18:3 and 18:3-16:0 PE will not condense with cholesterol in mixed monolayers at 22 C (1,2). Therefore we must attribute the condensation found here to the presence of other PE molecules, particularly those containing 20:4 or 22:6 (Table I). It seems probable that cholesterol can condense with many molecular species of PE, as it does with lecithin molecules of various structures, and that similar interactions could also occur in membranes known to contain both cholesterol and PE.

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REFERENCES

1. Van Deenen, L.L.M., Ann. N.Y. Acad. Sci. 137:717 (1966).
2. Demel, R.A., L.L.M. Van Deenen and B.A. Pethica, Biochim. Biophys. Acta 235:11 (1967).
3. Joos, R., and R.A. Demel, Ibid. 183:447 (1969).
4. Tinoco, J., and D.J. McIntosh, Chem. Phys. Lipids 4:72 (1970).
5. Demel, R.A., K.R. Bruckdorfer and L.L.M. Van Deenen, Biochim. Biophys. Acta 255:311 (1972).
6. Demel, R.A., W.S.M. Geurts Van Kessel and L.L.M. Van Deenen, Ibid. 266:26 (1972).
7. Ghosh, D., R.L. Lyman and J. Tinoco, Chem. Phys. Lipids 7:173 (1971).
8. Ghosh, D., and J. Tinoco, Biochim. Biophys. Acta 266:41 (1972).
9. Ghosh, D., M.A. Williams and J. Tinoco, Ibid. In press.
10. Firoiti, J.A., and R.J. Sims, JAOCS 44:221 (1967).
11. Smith, L.L., W.S. Matthews, J.C. Price, R.C. Bachman and B. Reynolds, J. Chromatogr. 27:187 (1967).
12. Fieser, L.F., and M. Fieser, "Steroids," Reinhold Publ. Corp., New York 1959, p. 28.
13. Skipski, V.P., R.F. Peterson and M. Barclay, Biochem. J. 90:374 (1964).
14. Tinoco, J., A. Shannon, P. Miljanich, R.L. Lyman and R. Okey, Analyt. Biochem. 3:514 (1962).
15. Vandenheuvel, F.A., JAOCS 40:455 (1963).
16. Kamel, A.M., N.D. Weiner and A. Felmeister, Chem. Phys. Lipids 6:225 (1971).
17. Standish, M.M., and B.A. Pethica, Trans. Faraday. Soc. 64:1113 (1968).
18. Hsia, J.C., and J.M. Boggs, Biochim. Biophys. Acta 266:18 (1972).

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The Structures of the Principal Glycerolipids of Pig Liver

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ABSTRACT

The lipid composition of pig liver has been determined. The principal glycerolipids, i.e., triglycerides, phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl inositol, were isolated and the positional distribution of fatty acids in each determined by stereospecific analysis procedures. Previous results for the triglycerides were confirmed, while the phospholipids were similar in structure to those found in most other animal livers. The triglycerides were separated into simpler molecular species by combinations of silver nitrate thin layer chromatography and high temperature gas liquid chromatography, but the proportions found did not agree well with those calculated assuming a 1-random, 2-random, 3-random arrangement. The phospholipids were hydrolyzed with phospholipase C and converted to diglyceride acetates that were fractionated into simpler molecular species by the same procedures as were used with triglycerides. Highly specific fatty acid combinations were found in molecular species, and these specificities were very similar to those reported in similar lipids from the livers of such disparate species as the rat and chicken.

INTRODUCTION

A considerable amount of information is now available on the structures of lipids from mammalian tissues (reviewed recently by Kuksis [1]), but most lipid analysts have concentrated on single specific lipid classes from a variety of tissues or species rather than systematically examining and comparing all the glycerolipids from a single source. The exception is the liver lipids of the rat which Wood and Harlow (2,3), in particular, have examined in some detail. Tumor lipids (4) have been studied in a similar manner and have important structural differences from those of normal tissues. The structures of all the lipid classes from many other tissues from a number of species must be determined and compared, if analytical studies are to make a significant contribution to a knowledge of the biosynthesis and interconversions of glycerolipids. As lipid metabolism in the pig is known to be different in many respects from that in other species, the structures of the main glycerolipids of pig liver have been determined and are now described.

MATERIALS AND METHODS

Animals

Six large white pigs, on standard diets, were slaughtered when the reached 90 kg live weight. The livers were removed and 500 g portions of each extracted with chloroform-methanol 2:1 v/v (36). Lipid extracts were pooled.

Lipid Quantification

Neutral lipid classes were separated by thin layer chromatography (TLC) on Silica Gel G layers (E. Merck, A.G., Darmstadt) and phospholipids on Silica Gel H layers (Camag, Muttenz), and the amounts and fatty acid compositions of each were determined by gas liquid chromatography (GLC) of the constituent fatty acids with methyl pentadecanoate added as internal standard. The procedure has been described in detail elsewhere (5,6).

TABLE I

Lipid Composition of Pig Liver

Total lipid classes[a]	Amount, wt %	Phospholipid classes	Amount, mol %
Cholesteryl esters	4.0	Phosphatidic acid-cardiolipin	6.0
Triglycerides	15.5	Phosphatidyl ethanolamine	21.3
Free fatty acids	1.9	Phosphatidyl serine	1.3
Diglycerides	0.2	Phosphatidyl inositol	7.2
Phospholipids	78.4	Phosphatidyl choline	57.5
		Sphingomyelin	4.5
		Lysophosphatidyl choline	2.1

[a]Free cholesterol was present but was not determined.

TABLE II

Positional Distribution of Fatty Acids in Glycerolipids of Pig Liver

Position	Fatty acid composition								
	16:0	16:1	18:0	18:1	18:2	20:3	20:4	20:5	22:5	22:6
Triglycerides	24.2	2.5	9.2	36.6	15.8	0.8	4.6	1.1	1.7	1.0
1	41.3	1.6	17.1	23.8	9.8	1.3	2.5	0.5	—	—
2	18.3	4.1	1.5	40.9	25.0	0.9	3.6	0.6	1.9	1.2
3	13.0	1.8	9.0	45.1	12.6	0.6	7.7	2.2	3.2	1.8
Phosphatidyl choline	19.3	0.8	26.2	13.0	16.0	1.2	10.9	3.4	3.5	3.6
1	37.0	0.6	53.3	4.7	1.3	—	0.3	0.1	—	—
2	2.2	0.6	0.3	21.4	30.6	2.6	20.8	6.2	6.2	8.2
Phosphatidyl ethanolamine	7.0	—	36.8	4.6	7.6	0.6	27.1	3.7	3.6	6.8
1	14.8	—	75.3	6.3	0.5	—	—	—	—	—
2	0.6	0.2	0.2	3.4	13.6	1.4	49.6	7.2	6.7	16.1
Phosphatidyl inositol	4.2	0.2	4.7	44.0	2.4	4.3	33.1	0.5	2.1	1.4
1	5.6	—	87.9	2.8	2.2	—	—	—	—	—
2	2.0	0.4	1.3	1.8	3.1	8.3	70.3	1.0	4.6	2.0

Gas Liquid Chromatography

Fatty acid analyses were performed on columns (7 ft x .25 in.) of 15% EGSS-X on Gas-chrom P (100-120 mesh; Applied Science Labs., Inc., State College, Pa.) isothermally at 180 C in a Pye 104 chromatograph. Components were identified by their retention times relative to authentic standards and by their chromatographic behavior on thin layers of Silica Gel G impregnated with silver nitrate. The amount of each ester present (wt%) was calculated from the product of the peak height and retention time, and results were converted to mol% by multiplying these values by appropriate factors.

Intact diglyceride acetates and triglycerides, after hydrogenation with platinum dioxide as catalyst (Adam's catalyst), were analyzed by GLC on columns (18 in. x .25 in.) of 1% SE 30 on Gas-chrom Q (100-120 mesh; Applied Science Labs., Inc.), temperature-programed from 250-300 C at 2 C/min (diglyceride acetates) or from 290-330 C at 1.5 C/min (triglycerides). Nitrogen at a flow rate of 50 ml/min was the carrier gas for all GLC analyses. The amount of each component (wt%) was determined with the aid of a Chromalog 2 integrator (Kent Instrument Co., Luton, Beds.) and results were converted to mol% by multiplying by appropriate factors. Isomers differing in the combined chain lengths of the fatty acid constituents by one carbon atom were separable.

Preparative Separation of Lipids

Triglycerides were obtained by preparative TLC on Silica Gel G layers (0.5 mm thick) with hexane-diethyl ether 80:20 v/v as developing solvent. Fractions rich in phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl inositol were obtained by chromatography on columns of DEAE cellulose prepared and eluted by the procedure of Rouser et al. (7). Pure phosphatidyl choline was obtained from the appropriate fraction by preparative TLC on Silica Gel G layers (0.5 mm thick) with chloroform-methanol-water 25:8:1 v/v as solvent system, pure phosphatidyl ethanolamine was obtained similarly, except that chloroform-methanol-water 80:20:0.5 v/v was the solvent system and pure phosphatidyl inositol was obtained by TLC on layers of Silica Gel H in which magnesium silicate (10% w/w) was incorporated (8); chloroform-methanol-7N ammonia 60:35:4 v/v was the developing solvent. Lipids were recovered from the adsorbents by elution with chloroform-methanol-water 5:5:1 v/v.

Enzymatic Hydrolyses of Lipids

The modified Brockerhoff procedure used

TABLE III

Principal (>1% of the Total) Molecular Species of Pig Liver Triglycerides

Species	Found	Calculated[a]	Species	Found	Calculated[a]
16:0-16:0-16:0	0.5	1.0	16:0-18:1-18:1	15.4	10.8
16:0-16:0-16:1	1.2	0.4	16:0-18:0-18:2	3.2	2.1
16:0-16:0-18:0	0.8	1.0	16:0-18:1-18:2	11.4	9.5
16:0-16:0-18:1	4.6	6.1	16:0-18:2-18:2	3.0	1.9
16:0-16:0-18:2	1.7	2.5	18:0-18:0-18:1	1.5	0.8
16:0-16:1-18:1	2.9	1.5	18:0-18:1-18:1	3.1	4.2
16:0-16:1-18:2	1.4	0.6	18:0-18:1-18:2	3.1	3.8
16:0-18:0-18:0	1.0	0.4	18:0-18:2-18:2	1.3	0.8
16:0-18:0-18:1	8.6	4.6	18:1-18:1-18:1	3.7	4.4
			18:1-18:1-18:2	2.9	5.7

[a]Assuming a 1-random, 2-random, 3-random arrangement.

for the stereospecific analysis of triglycerides has been described elsewhere (9,10). Results for position 1 were obtained by analysis of the lysophosphatide produced in the final stage of the procedure; those for position 2 were obtained by pancreatic lipase hydrolysis, and those for position 3 were calculated by difference from the known triglyceride composition. Checks were possible on the results for positions 2 and 3, and only those analyses that conformed to the standards of accuracy described earlier (9,10) were accepted.

Phospholipase A hydrolysis with the snake venom of *Ophiophagus hannah* was used to obtain the positional distribution of fatty acids in the phosphatidyl choline (11), the phosphatidyl ethanolamine (12) and the phosphatidyl inositol (12).

The phospholipase C of *Clostridium welchii* (Sigma Chemical Co., London) was used to prepare diglycerides from phosphatidyl choline (13) and that of *Bacillus cereus* (prepared by the method of Ottolenghi [14]) to prepare diglycerides from phosphatidyl ethanolamine (2) and phosphatidyl inositol (3). The acetate derivatives were prepared without delay (13) and were purified by preparative TLC on layers of Silica Gel G; hexane-diethyl ether 6:4 v/v was the developing solvent.

Silver Nitrate Chromatography

Separations were performed on 20 x 10 cm glass plates coated with a layer 0.5 mm thick of Silica Gel G impregnated with 10% (w/w) silver nitrate. Triglycerides were separated with hexane-diethyl ether-benzene-methanol 70:10:20:1 v/v as solvent system. Bands were visualized under UV light after spraying with 2',7'-dichlorofluorescein in methanol and were scraped into test tubes. When the fatty acid compositions of the fractions were required, a standard solution of methyl pentadecanoate in methanol (1 ml) was added, and the components were recovered by the procedure of Åkesson (15) for transesterification with sodium methoxide. When the intact fractions were to be analyzed by high temperature GLC, a standard solution of tritetradecanoin in diethyl ether (1 ml) was added and the components isolated as before. The amount of each fraction was determined by relating the combined areas of the various peaks on the GLC recorder chart to that of the appropriate internal standard.

Diglyceride acetates were separated with chloroform-methanol 98.5:1.5 v/v as developing solvent (species with 0 to 2 double bonds per molecule) or with chloroform-methanol 97:3 v/v as developing solvent (3 to 6 double bonds per molecule), and bands were recovered for analysis as above except that tridecanoin was added as an internal standard when the intact lipids were analyzed by high temperature GLC.

RESULTS AND DISCUSSION

Lipid Composition

The major lipid classes in pig liver were separated by TLC and determined by gas chromatography of the constituent fatty acids in the presence of a suitable internal standard. The results are listed in Table I. Triglycerides were the major simple lipid class present, but there were also appreciable amounts of cholesteryl esters. Phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl inositol were the most abundant phospholipids. Triglycerides, in sufficient quantity for structural studies, were isolated by preparative TLC. Larger quantities of the phosphatides were isolated by DEAE cellulose column chromatography followed by preparative TLC.

The fatty acid constituents of the lipids were those common to most mammalian tissues, viz., 16:0, 18:0 and 18:1, together with 18:2 and long chain polyunsaturated fatty acids of the

TABLE IV

Principal Molecular Species of Pig Liver Glycerophosphatides

Molecular species	Proportion in each phospholipid class, mol %					
	Found			Calculated[a]		
	PC	PE	PI	PC	PE	PI
16:0-16:0	1.2	—	—	0.8	0.1	0.1
-18:1	15.8	1.1	0.6	8.0	0.5	0.2
-18:2	12.8	2.3	1.0	11.4	2.0	0.2
-20:3[b]	1.3	0.2	0.4	1.0	0.2	0.5
-20:4	4.1	5.4	2.4	7.7	7.3	3.9
-20:5	2.0	1.2	0.3	2.3	1.1	0.1
-22:5&6	3.7	5.3	0.4	5.3	3.4	0.4
18:0-18:1	7.8	1.7	1.4	11.4	2.6	1.6
-18:2	20.8	10.9	4.5	16.3	10.2	2.8
-20:3[b]	1.9	0.3	11.9	1.4	1.0	7.3
-20:4	12.1	42.4	62.0	11.1	37.4	61.8
20:5	3.9	6.8	0.6	3.3	5.4	0.8
-22:5&6	6.9	11.1	5.6	7.7	17.2	5.9
18:1-18:1	0.6	1.5	0.3	1.0	0.2	0.1
-18:2	1.7	1.6	0.3	1.7	0.9	0.1
-20:4	0.6	2.3	0.4	0.7	3.1	1.9

[a]Assuming a 1-random, 2-random arrangement.
[b]Both the (*n*-6) and (*n*-3) isomers are present.

(*n*-6) and (*n*-3) families as detailed in Table II. If the main lipids are considered in the order triglycerides, phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl inositol, they contain decreasing amounts of 16:0, 18:1 and 18:2 and increasing amounts of 18:0 and 20:4 (*n*-6) together with the polyunsaturated fatty acids of the (*n*-3) series, except in the phosphatidyl inositol where these tend to be minor components. Similar results have been obtained with rat liver lipids (2,3). No alkyl- or alkenyl-derivatives of these lipids were detectable (16).

Positional Distribution of Fatty Acids in the Glycerolipids

The fatty acid composition of each of the three positions of the *sn*-glycerol moiety was determined by a stereospecific analysis procedure (9,10) based on that devised by Brockerhoff (17). The results (Table II) were very similar to those obtained in an earlier study (10) and are listed only for the sake of comparison with the analogous results for the phosphatides and need not be discussed further here. The positional distributions of fatty acids in each of the phospholipids were determined by hydrolysis with the stereospecific phospholipase A_2 (EC 3.1.1.4) of snake venom *(O. hannah)*, and the results are also listed in Table II. In all, as is true of most glycerophosphatides, saturated fatty acids were concentrated in position 1 and unsaturated in position 2, but there was much less of the saturated components in position 2 than is found in this position in the corresponding glycerophosphatides of rat (2,3) or rabbit (18) liver or indeed of other pig tissues, e.g., kidney (19), muscle (20), serum (21) and adipose tissue (21). The acyltransferases involved in the biosynthesis of phospholipids in pig liver must therefore have an even greater specificity for particular fatty acids than do the corresponding enzymes in other species or in other tissues of the pig.

There is little similarity between the results for the fatty acid compositions of positions 1 and 2 in the triglycerides, and those for the same positions in the phospholipids although diglycerides may be intermediates in the biosynthesis of all the glycerolipids. A similar finding was obtained with rat liver glycerolipids (2,3).

Molecular Species of Glycerolipids

The triglycerides were separated into simpler species first by silver nitrate TLC and then by high temperature GLC of the intact molecules, in order that a comprehensive picture of the molecular species separated by the overall degree of unsaturation and combined chain lengths of the fatty acid constituents might be obtained. The more important of these (>1%) are listed in Table III (it was not technically possible to adequately resolve molecules containing polyunsaturated fatty acids). Previous work in which species of pig liver triglycerides separated by silver nitrate TLC alone were subjected to stereospecific analysis had indicated that the proportions of the molecular species could be roughly predicted from the

positional distribution of fatty acids in the triglycerides if a 1-random, 2-random, 3-random arrangement was postulated (10). In this instance, although stereospecific analyses of the fractions were not performed, the data did not agree quite as well with this hypothesis. It may therefore be possible that the acylation of further positions of partially esterified intermediates in triglyceride biosynthesis may not be correlated with the degree of unsaturation of the fatty acids already esterified, but may be dependent on their chain lengths. The discrepancies in the results for rat liver triglycerides obtained by Slakey and Lands (22) and by Wood and Harlow (2) could be explained on this basis. The former separated species only according to their degree of unsaturation and found that the fatty acids were esterified with noncorrelative specificity; the latter separated species only according to their approximate molecular weights and found considerable specificity in the molecular species formed. It therefore appears to be important that analysts should not utilize only one property in separating molecular species of lipids. Even with the combinations of separatory procedures used in this study, it was not possible to adequately delineate the fatty acid specificities of the acyltransferases and it may be necessary to perform much more detailed analyses, such as those suggested by Hammond (23), before these specificities can be adequately defined.

The phospholipids, isolated by DEAE cellulose chromatography followed by preparative TLC, were hydrolyzed to diglycerides by the action of phospholipase C and converted to diglyceride acetates for separation into molecular species by combinations of methods analogous to those described above for triglycerides. The results are listed in Table IV.

Only the phosphatidyl choline contained large amounts of mono- and dienoic species, while the tetraenoic species assumed much greater significance in the phosphatidyl ethanolamine and phosphatidyl inositol. If the proportions of the various molecular species are calculated from the positional distributions of the fatty acids assuming a 1-random, 2-random arrangement, the actual and calculated results did not agree well, especially in the phosphatidyl choline and phosphatidyl ethanolamine. The discrepancies were smaller in magnitude in the phosphatidyl inositol, in which the amount of 16:0 was low and by far the major component was the single species 18:0-20:4 that comprised more than 60% of the total. The deviations from the calculated values were largely the result of preferential associations of 16:0 and 18:0 with specific unsaturated acids

TABLE V

Association of Palmitic and Stearic Acids with Unsaturated Fatty Acids in the Glycerophosphatides of Pig Liver

Unsaturated fatty acid	Degree of preference for palmitic acid[a]		
	PC	PE	PI
18:1	2.75	3.41	4.56
18:2	0.84	1.11	2.36
20:3	0.9	—	0.36
20:4	0.46	0.67	0.41
20:5	0.70	1.08	—
22:5/6	0.73	2.51	0.76

[a]Degree of preference for 16:0 = (16:0)/(18:0)(class)/(16:0)/(18:0)(total). (Nonpreferential association = 1.0 ± 0.1, assuming a relative error of 10%.) (24,25).

in molecular species, and these specificities can be seen better if the degree of preference for 16:0 in each species is calculated (Table V) (24,25). In all the phosphatides, 18:1 exhibited a marked preference for 16:0, the less 16:0 and 18:1 available, the greater the preference. Arachidonic acid was always preferentially associated with 18:0 in molecular species, but 18:2 did not exhibit a marked preference for either saturated acid except in the phosphatidyl inositol where it was associated with 16:0 to a greater extent than might have been anticipated. The polyunsaturated components 22:5 (*n*-3) and 22:6 (*n*-3), which were not resolved from each other, showed some degree of preference for 18:0 in the phosphatidyl choline and phosphatidyl inositol but a marked preference for 16:0 in the phosphatidyl ethanolamine. Similar associations of specific fatty acids in pairs are known to occur in rat liver (2,26,27) and egg yolk (24) phospholipids, although a 1-random, 2-random arrangement of fatty acids was found in the molecular species of tumor phospholipids (4).

Although the positional distributions of fatty acids in the diglyceride acetate fractions were not determined by pancreatic lipase hydrolysis, the proportions of molecular species of the type 1-unsaturated, 2-saturated must be very small as in the phosphatidyl choline; for example, 75% of the saturated fatty acids in position 2 were in the disaturated fraction.

Although the structures of lipids (especially the triglycerides from other pig tissues) are very different from those of most other species, the structures of the phospholipids of pig liver are very similar to those of rat liver (2,26,27) and to those of egg yolk (24) which may also originate in the liver of the chicken (28). For example, the preferential association of 22:6 (*n*-3) with 16:0 in the phosphatidyl ethanol-

amine and with 18:0 in the phosphatidyl choline, revealed in this study, has also been found in the rat and chicken. When the livers of such disparate species as pig, rat and chicken exhibit such similar specificities in lipid biosynthesis, it would appear that the enzyme systems in all must be very similar or that the structural requirements for certain types of molecular species of phospholipids in all are the same. The triglycerides bore very little similarity in the positional distributions of fatty acids in positions 1 and 2 to those of the phospholipids in the liver of the pig, and similar findings have been obtained with other species of animals; but this cannot be regarded as surprising, since deacylation-reacylation appear to play a large part in the biosynthesis of particular molecular species of phospholipids (29-32). Recently it has been shown that phosphatidyl cholines (33,34) and triglycerides (10,35) from different tissues in the same animal can be very different in structure, but the structural relationships of such lipids to each other or to the other lipid classes in tissues other than liver have yet to be adequately defined.

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REFERENCES

1. Kuksis, A., in "Progesss in the Chemistry of Fats and Other Lipids," Vol. 12, Edited by R.T. Holman, Pergamon Press, London, 1972, p. 1.
2. Wood, R., and R.D. Harlow, Arch. Biochem. Biophys. 131:495 (1969).
3. Wood, R., and R.D. Harlow, Ibid. 135:272 (1969).
4. Wood, R., and F. Snyder, Ibid. 131:478 (1969).
5. Christie, W.W., R.C. Noble and J.H. Moore, Analyst 95:940 (1970).
6. Christie, W.W., Ibid. 97:221 (1972).
7. Rouser, G., G. Kritchevsky and A. Yamamoto, in "Lipid Chromatographic Analysis," Vol. 1, Edited by G.V. Marinetti, Edward Arnold Ltd., London, 1967, p. 99.
8. Rouser, G., C. Galli, E. Lieber, M.L. Blank and O.S. Privett, JAOCS 41:836 (1964).
9. Christie, W.W., and J.H. Moore, Biochim. Biophys. Acta 176:445 (1969).
10. Christie, W.W., and J.H. Moore, Ibid. 210:46 (1970).
11. Robertson, A.F., and W.E.M. Lands, Biochemistry 1:804 (1962).
12. Van Golde, L.M.G., and L.L.M. Van Deenen, Chem. Phys. Lipids 1:157 (1967).
13. Renkonen, O., JAOCS 42:298 (1965).
14. Ottolenghi, A.C., in "Methods in Enzymology," Vol. XIV, Edited by J.M. Lowenstein, Academic Press, New York, 1969, p. 188.
15. Åkesson, B., Eur. J. Biochem. 9:463 (1969).
16. Wood, R., and F. Snyder, Lipids 3:129 (1968).
17. Brockerhoff, H., J. Lipid Res. 6:10 (1965).
18. Moore, J.H., and D.L. Williams, Biochim. Biophys. Acta 98:137 (1965).
19. Hagen, P-O., Lipids 6:935 (1971).
20. Kuchmak, M., and L.R. Dugan, JAOCS 42:45 (1965).
21. Grigor, M.R., M.L. Blank and F. Snyder, Lipids 6:965 (1971).
22. Slakey, P.M., and W.E.M. Lands, Ibid. 3:30 (1968).
23. Hammond, E.G., Ibid. 4:246 (1969).
24. Holub, B.J., and A. Kuksis, Ibid. 4:466 (1969).
25. Holub, B.J., A. Kuksis and W. Thompson, J. Lipid Res. 11:558 (1970).
26. Holub, B.J., and A. Kuksis, Can. J. Biochem. 49:1347 (1971).
27. Arvidson, G.A.E., Eur. J. Biochem. 4:478 (1968).
28. Christie, W.W., and J.H. Moore, Comp. Biochem. Physiol. 41B:287 (1972).
29. Lands, W.E.M., and P. Hart, J. Lipid Res. 5:81 (1964).
30. Kanoh, H., Biochim. Biophys. Acta 176:756 (1969).
31. Holub, B.J., W.C. Breckenridge and A. Kuksis, Lipids 6:307 (1971).
32. Vereyken, J.M., A. Montfoort and L.M.G. Van Golde, Biochim. Biophys. Acta 260:70 (1972).
33. Kuksis, A., L. Marai, W.C. Breckenridge, D.A. Gornall and O. Stachnyk, Can. J. Physiol. Pharm. 46:511 (1968).
34. Montfoort, A., L.M.G. Van Golde and L.L.M. Van Deenen, Biochim. Biophys. Acta 231:335 (1971).
35. Christie, W.W., and J.H. Moore, J. Sci. Food Agr. 22:120 (1971).
36. Folch, J., M. Lees and G.H.S. Stanley, J. Biol. Chem. 226:497 (1957).

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Phosphatidylethanolamine Metabolism in Rats Fed a Low Methionine, Choline-Deficient Diet[1]

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ABSTRACT

The metabolism of phosphatidylethanolamine (PE) was studied in male rats fed a low methionine diet for 7 days with or without supplemental choline. Groups of animals were injected with 2-^{14}C-ethanolamine and killed at intervals thereafter up to 72 hr. Liver phospholipids were isolated, and PE and phosphatidylcholine (PC) were separated by argentation chromatography into diene (18:2), tetraene (20:4) and hexaene (22:6) fractions. Fatty acid composition and the distribution of radioactivity and specific activity in the total phospholipids and in the fractions were determined. Choline deficiency did not affect total liver phospholipid, but it did increase the amount of PE and decreased that of PC. The major effect of the deficiency on phospholipid fatty acids was to decrease the proportion of PE arachidonate and to increase that of docosahexaenoate. Ethanolamine incorporation into liver PE of deficient rats was only slightly less than in the controls, but loss of the radioactivity from the PE was slower. Ethanolamine radioactivity appearing in the PC of deficient rats was about half that of the controls, even though specific activities of the PE precursors were similar to the control rats. Choline deficiency increased the biological half-lives of the total PE and its fractions. Although the proportion of PE tetraenoic fraction was reduced, the total amount of this liver PE fraction in deficient rats was not affected. However the amount of hexaenoic fraction was doubled, and it accounted for most of the increased quantity of liver PE seen in deficient animals. The results suggested that in choline deficiency PE synthesis was delayed but not appreciably suppressed, and that limited availability of methionine for methylating the PE fractions in their proper proportions affected the concentrations of the PE fractions and impaired their normal conversion to PC.

INTRODUCTION

It is generally accepted that the only pathway for de novo synthesis of choline in the rat is via methylation of phosphatidylethanolamine (PE) to form phosphatidylcholine (PC). Therefore it is not surprising that a number of investigators have reported the utilization of

[1]Presented in part at the AOCS Meeting, Houston, May 1971.

TABLE I

Liver Lipids in Rats Fed Diets with and without Choline

A.

Diet	Liver[a], g	Total lipid[a], %	Phospholipid, mg/liver
Control	8.50 ± 0.14	3.89 ± 0.01	270 ± 4
Choline-deficient	10.8 ± 0.3	17.3 ± 1.2	266 ±5

B.

Diet	Distribution of phospholipids, %[b]			
	Sph	PC	PE	PI+PS
Control	10.7 ± 0.2	48.8 ± 0.2	23.0 ± 0.3	11.8 ± 0.2
Choline-deficient	10.7 ± 0.5	42.8 ± 0.7	28.5 ± 0.6	13.1 ± 0.4
P value		<.01	<.01	<.05

[a]Wet wt of liver. Means ± standard error of 20 to 24 rats in each diet group.

[b]Sph = sphingomyelin; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PI+PS = phosphatidylinositol and phosphatidylserine combined.

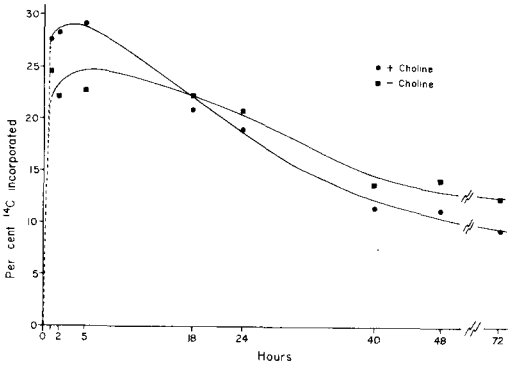

FIG. 1. Per cent of injected 1,2-^{14}C-ethanolamine incorporated into liver phospholipids of rats fed diets with and without choline. Each point on a curve represents a sample pooled from three rats.

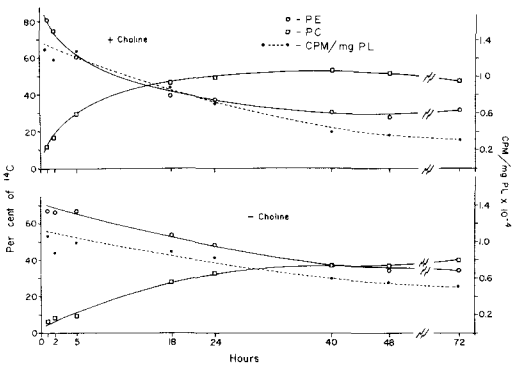

FIG. 2. Specific activity of total liver phospholipids and distribution of radioactivity from 1,2-^{14}C-ethanolamine between liver phosphatidylethanolamine and phosphatidylcholine in rats fed diets with and without choline. Phospholipid was calculated by multiplying mg P x 25. Each point on a curve represents a sample pooled from three rats.

this pathway to be affected when an animal is fed a choline deficient diet. Agreement is lacking, however, on whether choline deficiency stimulates or suppresses the pathway. For example, Lombardi et al. (1), in very short term experiments (rats fed deficient diets for only 15 hr), reported increased incorporation of the labeled methyl group of methionine into the liver PC. In 2 day-deficiency experiments, Thompson et al. (2) also demonstrated increased uptakes of methionine methyl group by rat liver PC, and Glenn and Austin (3) showed increased methylation of PE to PC in rats made deficient in choline for two weeks. In these experiments it appeared that length of the deficiency did not influence the pathway, as was proposed by Yamamoto et al. (4). In contrast to these results, however, are those of Blumenstein (5) who, in 2 day-deficient rats, demonstrated an early reduction in the incorporation of ^{14}C-label from ethanolamine or methionine methyl groups into PC. Results obtaned by Haines and Rose (6) also indicated that in choline-deficient rats, incorporation of activity from ^{14}C-ethanolamine into PE and PC was reduced. Although as yet no specific explanation is evident for these divergent results, the age of rats, interval on deficient diet, time of sacrifice after injecting the labeled substance, and amount and kind of protein in the diet are probably contributing factors.

Beare-Rogers (7) reported a reduced proportion of arachidonic acid and increased docosahexaenoic acid in liver PE of choline-deficient rats as compared with controls, and speculated on the possibility that a subspecies of PE (possibly that containing arachidonic acid) necessary for the synthesis of PC may not be synthesized or be available in the choline-deficient rats. Lombardi et al. (1) and Day and Levy (8) have also suggested that lack of or shift in the concentration of a specific PC species may impair synthesis and release of lipoprotein from the liver, leading possibly to the accumulation of hepatic triglyceride, characteristic of a choline deficiency in rats.

In recent investigations with choline-deficient rats (9), we have also found the changes in rat liver PE arachidonic and docosahexaenoic acids shown by Beare-Rogers. In addition, we found that choline-deficient rats converted more methyl-labeled methionine into a subspecies of liver PC rich in docosahexaenoic acid than did controls. Consequently, in order to see whether choline deficiency altered the metabolism of certain phosphatide fractions, we conducted, in rats fed diets with and without choline, a time study of incorporation and disappearance of radioactive ethanolamine in three main fractions of liver PE and PC.

MATERIALS AND METHODS

Animals

Male Long-Evans rats, weighing ca. 175 g, were fed ad libitum for 7 days either a control diet containing 20% soybean protein and 0.3% choline chloride or the same diet deficient in choline. Details of the diet composition have been described previously (9). The animals were allowed free access to food and water until they were killed.

During an experiment, three rats from each diet group at each time period were injected intraperitoneally with 3.00 μCi of 1,2-^{14}C-ethanolamine (International Chemical and Nuclear Co., Irvine, Calif.) per 100 g body wt. They were then anesthetized (Diabutal, Diamond

Lab., Des Moines, Iowa) and killed by withdrawal of blood directly from the heart. Livers were removed, frozen on solid CO_2 and lyophilized.

Lipid Analysis

Liver lipids were extracted with chloroform-methanol containing hydroquinone as described previously (9). During subsequent procedures, lipids were always kept in solution or under nitrogen in order to minimize oxidation. Solvents and chemicals were of reagent grade purity. The liver lipid extracts were analyzed for total radioactivity, total phosphorus (10) and total lipid (11). The liver phospholipids were isolated from neutral lipids (in both diet groups) by preparative thin layer chromatography (TLC) (12) to avoid complications caused by high concentrations of triglycerides in the deficient livers. Phospholipid classes were then separated by the TLC system of Skipski et al. (13). Considerable radioactivity was found in a diffuse band that migrated between PE and PC. This material, "fraction 3," appeared to be a lipid and was not characterized further. Its specific activity is shown in Figure 3. Phospholipid bands were scraped from the plate into 15 ml centrifuge tubes containing 10 ml methanol and 3 drops sulfuric acid (insufficient to interfere with subsequent phosphorus determination) and refluxed gently for 30-60 min. The silica gel was then eluted with absolute methanol and aqueous methanol, and the volume of eluate was reduced. The methyl esters were then extracted into petroleum ether for analysis by gas chromatography (GLC). The aqueous phase was analyzed for phosphorus (10). Plate blanks were analyzed for phosphorus and fatty acid content. Values were very low compared with the size of the sample itself. Fatty acid to phosphorus ratios obtained from these determinations were quite close to the theoretical mole ratios, which indicated that there was no significant loss of fatty acids due to oxidation during the manipulations.

Fractionation of PE and PC

After the separation of phospholipids from neutral lipid, PE and PC from individual animals were prepared by the TLC procedure of Skipski et al. (13). Equal amounts of PE or PC from at least three animals were pooled, and an aliquot of the pool was fractionated by argentation TLC as previously described (9,14,15).

Fatty Acid Analyses

Fatty acids of the phospholipid classes and fractions were analyzed with an Aerograph Model 200 gas chromatograph equipped with hydrogen flame detectors. The instrument was calibrated daily with a standard containing known proportions of saturated fatty acid methyl esters and methyl oleate, linoleate and arachidonate (Calbiochem, Los Angeles, Calif.; Hormel Institute, Austin, Minn.). Methyl esters of docosapentaenoic and docosahexaenoic acids were identified by their relative retention times before and after hydrogenation, and by comparison with an authentic sample of 22:6 methyl ester (Hormel Institute). Methyl esters were prepared by transesterification (14). The weights of fatty acids in the phospholipid fractions were obtained by GLC with the use of heptadecanoic acid as an internal standard (16). These weights, together with the radioactivity of the fraction, gave a specific activity expressed as cpm/μg fatty acid. This method was more sensitive and reproducible than determination of phosphorus in the relatively small samples at our disposal.

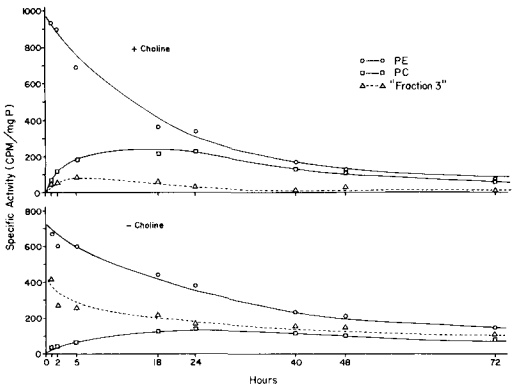

FIG. 3. Specific activities of liver phosphatidylethanolamine and phosphatidylcholine after administration of 1,2-^{14}C-ethanolamine to rats fed diets with and without choline. Each point on a curve represents a sample pooled from three rats.

Radioactivity Measurements

Radioactivities of the various lipid fractions were determined with a Beckman Model LS-100 liquid-scintillation counter. Lipid extracts were evaporated to dryness and counted in a solution of 0.4% 1,5-diphenyloxazole in toluene. Bands of phospholipid separated by TLC were scraped into counting vials and counted in a solution of 0.4% 2,5-diphenyloxazole in dioxane containing 10% naphthalene and diluted with 0.2 volume of water. Counting efficiency of ^{14}C in the dioxane mixture was 65%; in the toluene mixture it was 76%.

Statistical comparisons were made using the *t* test described by Snedecor (17).

RESULTS

Liver lipid analyses are shown in Table I. As

TABLE II

Fatty Acid Composition of Liver PE and PC from Rats Fed Diets with and without Choline

Diet	Fatty acid, wt %[a]						
	16:0	18:0	18:1	18:2	20:4	<20:4[b]	22:6
				Phosphatidylethanolamine			
Control	13.4 ±0.3	26.9 ±0.6	3.8	8.9 ±0.4	30.9 ±0.7	5.4	8.3 ±0.3
Choline deficient	17.7 ±0.6	31.0 ±0.8	2.4	6.2 ±0.4	21.3 ±0.8	6.6	13.6 ±0.6
P value	<.01	<.01	N.S.	<.01	<.01	N.S.	<.01
				Phosphatidylcholine			
Control	18.1	22.3 ±0.3	4.1 ±0.1	17.1 ±0.3	30.1 ±0.4	2.3	3.4
Choline deficient	18.0	26.9 ±0.6	3.0 ±0.2	14.0 ±0.6	28.5 ±0.7	1.6	3.6
P value	N.S.	<.01	<.01	<.01	N.S.	N.S.	N.S.

[a]Values are from 20 to 24 rats in each diet group. Minor fatty acids have been omitted.
[b]Consists mainly of 20:5 and 22:5 fatty acids.

expected, choline deficiency induced a severe fatty liver, although total phospholipids in the livers of the two groups of rats were the same. The proportion of PC in deficient rats was significantly reduced, while that of PE (as well as a small mixed fraction of PI and PS) was increased. Thus there was an absolute increase in the amount of PE and a decrease in that of PC. Similar results have been reported previously (6,7,9,18).

Figure 1 shows the incorporation of ethanolamine into liver phospholipids. Incorporation of the 1,2-^{14}C-ethanolamine took place rapidly in both groups, but was slightly lower in deficient rats until 18 hr after injection. The loss of radioactivity from the phospholipids of these animals appeared to be slower than in the controls.

The specific activities of total liver phospholipids and the distribution of radioactivity between PE and PC are shown in Figure 2. Control rats had over 80% of the total liver phospholipid radioactivity in the PE at 1 hr. With time, the proportion of label in the PE decreased, while that in the PC increased, so that by 15 hr both phospholipids were equally labeled. From that time to the end of the experiment ca. 50% of the phospholipid radioactivity remained in the PC, while PE maintained 30-35%. In deficient animals the loss of radioactivity from the PE was slower, and the proportion of radioactivity in the PC never rose above 40%.

Figure 3 shows the specific activities of the PE and PC from control and deficient animals. The specific activity of PE was higher in control rats for the first 18 hr of incorporation, and from then on the control and deficient rats had about equal specific activities. The specific activity of PC from both groups behaved similarly but was much lower in the deficient rats and reached a maximum later than in the controls. In deficient rats, "fraction 3" had a relatively high specific activity at 1 hr, which declined as the specific acitivity of the PC increased. In controls, however, this fraction never contained more than 5% of the total radioactivity.

Fatty acids of the liver PE and PC are shown in Table II. The effect of choline deficiency was most noticeable in the PE, where the proportion of arachidonic acid was decreased, while that of the docosahexaenoic acid was significantly increased. Less pronounced changes in linoleic, stearic and palmitic acid also were evident in this phospholipid. The fatty acid composition of the PC was relatively unaffected by choline deficiency, except for a decrease in the proportion of 18:2 and an increase in 18:0. Similar observations have been made by Beare-Rogers (7) on rats deficient in choline for 3 weeks.

Changes in the proportions of unsaturated fatty acids in the phosphatides represent changes in the proportions of their molecular species. Table III shows the fatty acid compositions and proportions of the PE fractions obtained after separation, according to unsaturation, by argentation chromatography. Resolution of the fractions agreed well with that of Arvidson (19) and with previous results obtained from rats fed purified diets similar to

TABLE III

Fatty Acids of Liver Phosphatidylethanolamine (PE) Fractions from Rats Fed Diets with or without Choline[a]

	+ Choline			– Choline		
PE fraction	22:6	20:4	18:2	22:6	20:4	18:2
Fatty acid	Fatty acid, wt %					
16:0	19.9	10.8	23.6	20.4	14.1	23.3
18:0	17.9	32.8	25.2	22.9	33.9	30.7
18:1	2.6	2.7	7.4	2.3	1.6	5.3
18:2	1.4	0.8	43.4	1.1	0.6	40.2
20:4	12.4	44.5	0	9.7	40.7	0
<20:4[b]	6.2	5.4	0	6.7	7.0	0
22:6	38.9	2.3	0	35.4	0.5	0
Total PE, %	19.7	67.4	12.9	39.6	49.2	11.3

[a]Values are averages of at least seven separate pools.
[b]Consists mainly of 20:5 and 22:5 fatty acids. Minor fatty acids have been omitted.

those in the present experiment (20). The tetraenoic (20:4) fraction predominated in both control and deficient groups. Choline deficiency reduced the proportion of this subfraction and doubled that of the hexaenoic (22:6) fraction. The composition and distribution of PC fractions were similar to those obtained previously from rats fed the same diets under the same conditions (9), so are not shown here.

Distribution of radioactivity in the PE fractions of control and choline-deficient rats is shown in Figure 4. In both control and deficient animals, the tetraenoic fractions rapidly gained radioactivity until they had attained ca. 60% of the total radioactivity. The hexaenoic fraction in both groups also quickly gained a high proportion of radioactivity. Control animals lost about half their initial acitivity by 18 hr after administration of the ethanolamine. In deficient animals, however, the hexaenoic fraction maintained its high proportion of label for a much longer period. The proportion and pattern of labeling in the dienoic (18:2) fraction was relatively low and similar for both groups of animals.

In order to see whether the turnover of the individual PE fractions was affected by choline deficiency, specific activities of the total PE and each of its fractions were plotted on a logarithmic scale vs. time and the regression lines that the points described were used to determine biological half-lives. These plots are shown in Figure 5. The calculated turnover time (1.44 $T_{1/2}$) of 30 hr for total PE in control animals corresponds with a 32 hr turnover time reported by Ansell and Hawthorne (21). In control animals, the hexaenoic and dienoic fractions had the shortest half-life, whereas the tetraenoic fraction had the longest. The rates of turnover of the PE fractions relative to each other were maintained in the choline-deficient animals, but the half-life of each fraction was considerably increased compared with control animals.

Figure 6 shows the specific activities of the three major fractions of PC. The proportions of the PC fractions were similar to those obtained earlier (9) and were not altered appreciably by the choline deficiency. Estimations of the half-life of these fractions were not made, since a log plot vs. time produced a biphasic curve which could not be resolved satisfactorily. Similar two-component curves for PC turnover have been reported by Pasternak and Friedrichs (22) and Bailey et al. (23). In both control and choline-deficient animals, the hexaenoic fraction attained the highest specific activity, reaching a maximum ca. 5 hr after administration of the labeled ethanolamine. Maximum specific activities of the dienoic and tetraenoic fractions

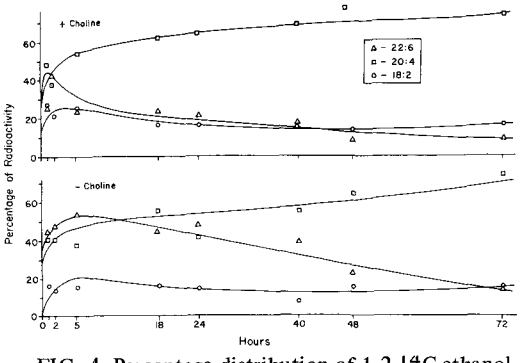

FIG. 4. Percentage distribution of 1,2-^{14}C-ethanolamine radioactivity in fractions of liver phosphatidylethanolamine from rats fed diets with and without choline. Dienoic = 18:2 = o; tetraenoic = 20;4 = □; hexaenoic = 22:6 = Δ. Each point on a curve represents a sample pooled from three rats.

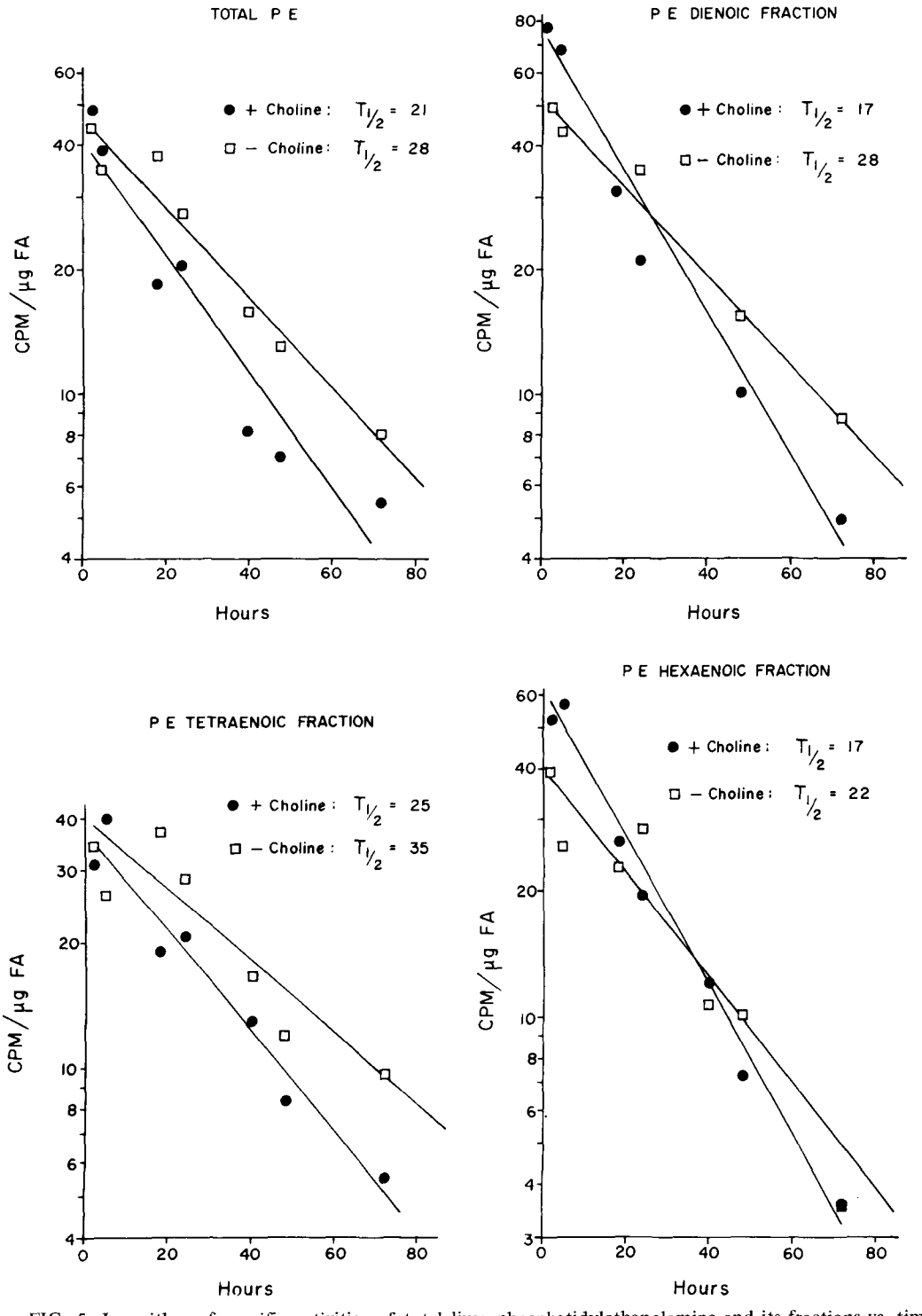

FIG. 5. Logarithm of specific activities of total liver phosphatidylethanolamine and its fractions vs. time. $T_{1/2} = \frac{0.693}{K}$; where K = slope of regression line (determined by method of least squares). Each point represents a sample pooled from three rats.

were not achieved, however, until ca. 20 hr after injection of the labeled compound. Although the pattern of incorporation of the ethanolamine label into PC fractions was the same regardless of diet, deficient animals incorporated only about half as much radioactivity as did animals fed choline. This was so, even though the initial specific activities of the PE fractions were similar for both groups (Fig. 5).

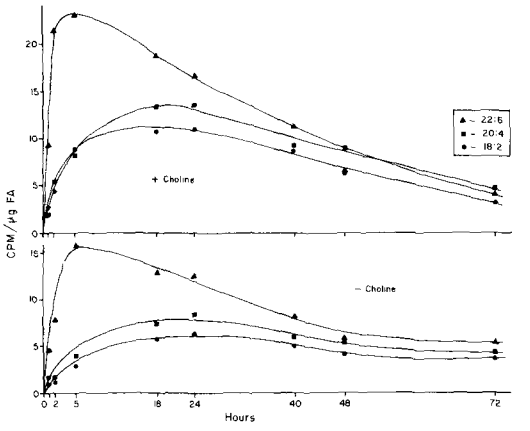

FIG. 6. Specific activities of liver phosphatidylcholine subfractions after injection of 1,2-14C-ethanolamine into rats fed diets with and without choline. Dienoic = 18:2 = ●; tetraenoic = 20:4 = ■; hexaenoic = 22:6 = ▲. Each point represents a sample pooled from three rats.

DISCUSSION

When an animal is fed a choline-deficient, low methionine diet, one might expect the pool sizes of choline, methionine and related metabolites to be altered. If the pool size of a particular compound is reduced, then incorporation of a radioactive dose of this compound may appear to be increased because the dose is diluted less than a normal amount. Similarly, if the pool size is increased, there may be a decrease in the amount of activity incorporated. These effects can appear even when there is no change in enzyme activity in the reactions involving the labeled compound. Increases and decreases in amount of radioactive incorporation can be due solely to the amount of endogenous substrate available to dilute the dose. In the case of an increased pool size of a precursor, if the label is not metabolized by other paths, it may remain available to the enzyme systems incorporating the label, which results in delayed but still high incorporation. Haines and Rose have reported that choline deficiency (3 day duration) increases the liver concentrations of ethanolamine, ethanolamine phosphate, cytidinediphosphoethanolamine, glycerylphosphorylethanolamine and PE (6). Under these circumstances one would expect delayed and possibly reduced incorporation of ethanolamine into PE. Figures 1 and 3 clearly show that choline deficiency did delay, but did not appreciably decrease, the incorporation of labeled ethanolamine into rat liver phospholipids or PE. In the short periods of incorporation studied by Haines and Rose (4-32 min) or Blumenstein (5) (10-80 min), choline-deficient rats incorporated far less ethanolamine into PE than controls did, but the incorporation curve of the deficient rats was rapidly rising and had not yet reached a maximum in either instance. The results of those short term experiments, along with our data for longer periods of incorporation, indicate that choline deficiency causes a delay in the incorporation of radioactive ethanolamine into rat liver PE, but the amount of incorporation is not greatly reduced by the deficiency. This result is consistent with the fact that the pool sizes of PE precursors are increased in choline deficiency (6). These increased pools would be expected to dilute the radioactivity, so that fewer counts would be incorporated per unit time, but the amount of ethanolamine (labeled plus endogenous) incorporated might be equal to, or greater than normal. Furthermore the labeled material, if it remained in the pool, could eventually become incorporated. This prolonged incorporation can be seen in our choline-deficient rats (Fig. 1).

Because so little of the ethanolamine radioactivity appeared in PC, both Haines and Rose (6) and Blumenstein (5) concluded that the methylation pathway for PC synthesis was not of much quantitative significance in choline deficiency. From our results (Fig. 2 and 3), it is clear that an appreciable proportion of ethanolamine eventually appears in the PC of both the control and deficient animals, but it is 2 hr or longer following administration of the ethanolamine that the labeling of the PC becomes apparent. Therefore it is not surprising that the other authors (5,6), because of their short term experiments, concluded that little conversion of PE to PC took place. However, even though deficient rats did convert ethanolamine phosphatides into PC, this synthesis appeared to be only about half that of controls (Fig. 6). Rytter and Cornatzer (24) have also reported reduced incorporation of radioactive ethanolamine into hepatic microsomal PC in rats fed choline-deficient diets, which were much more deficient in methyl groups than the diets used in the present experiment. Thus it appears that choline deficiency not only delays incorporation of ethanolamine into PE, but impairs the conver-

TABLE IV

Rates of Turnover of Total Liver PE and PE Fractions in Control and Choline-Deficient Rats

Phosphatidylethanolamine fraction	Amount of fraction, mg/liver	Half-life ($T_{1/2}$), hr	Turnover rate, mg/hr
	+ Choline		
Hexaenoic (22:6)	12.2	17	0.36
Tetraenoic (20:4)	41.8	25	0.84
Dienoic (18:2)	8.0	17	0.24
		Sum of fractions	1.44
Total PE	62.1	21	1.48
	− Choline		
Hexaenoic (22:6)	30.0	22	0.68
Tetraenoic (20:4)	37.3	35	0.53
Dienoic (18:2)	8.6	28	0.15
		Sum of fractions	1.36
Total PE	75.8	28	1.35

sion of PE to PC as well.

Although one can determine the rate of formation of a metabolite in vivo if the pool size and specific activity of its immediate precursor are known, we chose to measure the rate of loss of activity from total PE and its fractions. This rate of loss of radioactivity is the result of all the possible metabolic paths that either remove or incorporate radioactivity into PE. If recyling of the label occurs, radioactivity will reappear in PE, and this will reduce its rate of loss. Therefore the total rate of loss may be slower than the rate of the reaction that first removes the label, but it cannot be faster. From the apparent rate of removal of a PE fraction, one can infer that the rate of its formation must be either the same, higher, or lower, depending on whether the amount of the fraction remains the same, increases, or decreases. If the rate of removal does not change, yet the amount of metabolite (PE) increases, then it is reasonable to infer that its rate of formation must have increased. If, in choline deficiency, the rate of turnover (loss of radioactivity of a PE fraction) increases while the amount remains the same or increases, it is probably that the rate of formation of this fraction has increased.

From the biological half-lives and the amounts of the different PE fractions present in the livers, an estimate of the rates of disappearance of the fractions can be calculated as mg/hr (Table IV). For example, if half the total PE in control rats (62.1 mg) is turned over in 21 hr, this amounts to a turnover rate of 1.48 mg/hr. The table shows that total PE is turned over slightly more slowly in deficient rats than in controls, as is also shown in Figure 5. Choline-deficient rats had more total PE in their livers than did controls, and this accumulation is largely accounted for by the hexaenoic fraction, which was increased to more than double the control value (Table IV). Surprisingly, its rate of turnover is much higher in deficient rats. Therefore the rate of formation of hexaenoic PE must have been increased in deficient rats, so that accumulation of this fraction occurred despite an increased rate of removal. The increased turnover of the hexaenoic fraction might indicate an increased rate of conversion of hexaenoic PE to hexaenoic PC, but hexaenoic PC was not significantly increased in livers of choline-deficient rats as shown in this experiment (Table II) and by others (7, 25-27). Therefore the catabolism of a large portion of hexaenoic PE must have occurred by some other path, possibly by conversion to glyceryl-phosphorylethanolamine, which has been shown to accumulate in the livers of choline-deficient rats (6).

There was only a small change in the amounts of tetraenoic PE or dienoic PE due to choline deficiency. The deficiency produced decreases in their rates of catabolism, but since accumulation did not occur, it is probable that choline deficiency also reduced their rates of formation. Figure 4 shows that these two liver PE fractions did take up the labeled ethanolamine more slowly in deficient rats than in controls.

These results indicate, that while total incorporation of ethanolamine into liver PE of choline-deficient rats is not seriously reduced compared with the controls, the distribution of the utilization of ethanolamine in the PE fractions has been changed. The reasons for the change, especially for the great increase in PE hexaenoic fraction, are still not clear. However, since this PE fraction is normally rapidly methylated and converted to PC (20), it sug-

gests that the altered metabolism by choline-deficient animals may be related to an insufficient or not readily available supply of methyl groups.

In the present experiment, choline was absent from one diet, and methionine was about half its required level in both. There was conversion of PE to PC, but the conversion seemed to be impaired, and the amount of liver PC was reduced in choline deficiency. A recent report by Beare-Rogers (25) showed that dietary methionine will maintain the liver PC at a normal level even more effectively than dietary choline, although the number of potential methyl groups provided by the methionine was much lower than that provided by choline. Dietary methionine also had its largest effect on the arachidonic acid in liver PC. The increase in tetraenoic PC upon methionine feeding might be expected, since this PC fraction has been shown to be synthesized to a considerable extent by the methylation of tetraenoic PE (26-28). The availability of methyl groups to methylate this PE fraction may be a controlling factor in the amount of PC of a definite composition that can be synthesized in these animals. It is of some interest that the choline deficiency did not appreciably reduce the amount of the tetraenoic fraction in the liver PE (Table IV). Therefore the significantly lower proportion of PE arachidonic acid seen in these animals (Table II) is due mainly to the increased proportions of fatty acids contributed by the hexaenoic fraction and does not reflect a defect in the formation of tetraenoic PE.

Since the studies were conducted in animals with a fully developed fatty liver, they provide little direct information on how the methylation pathways of PC synthesis may be related to hepatic fat accumulation in choline-deficient rats. However the importance of the tetraenoic fractions of PE and PC in producing liver lipid accumulation has been indicated by the inverse relationship between per cent of arachidonate in liver phospholipids and the accumulation of hepatic triglyceride in choline-deficient rats reported by Beare-Rogers (29).

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REFERENCES

1. Lombardi, B., P. Pani, F.F. Schlunk and C. Shi-Hua, Lipids 4:67 (1969).
2. Thompson, W., G. MacDonald and S. Mookerjea, Biochim. Biophys. Acta 176:306 (1969).
3. Glenn, J.L., and W. Austin, Ibid. 231:153 (1971).
4. Yamamoto, A., M. Sano and M. Isozaki, J. Biochem. 65:85 (1969).
5. Blumenstein, J., Can. J. Physiol. Pharmacol. 46:487 (1968).
6. Haines, D.S.M., and C.J. Rose, Can. J. Biochem. 48:885 (1970).
7. Beare-Rogers, J.L., Ibid. 47:257 (1969).
8. Day, C.E., and R.S. Levy, Biochem. Med. 3:177 (1969).
9. Lyman, R.L., G. Sheehan and J. Tinoco, Can. J. Biochem. 49:71 (1971).
10. Sumner, J.B., Science 100:413 (1944).
11. Bragdon, J.H., J. Biol. Chem. 190:513 (1951).
12. Bowyer, D.E., W.M.F. Leat, A.N. Howard and G.A. Gresham, Biochim. Biophys. Acta 70:423 (1963).
13. Skipski, V.P., R.F. Peterson and M. Barclay, Biochem. J. 90:374 (1964).
14. Tinoco, J., S.M. Hopkins, D.J. McIntosh and G. Sheehan, Lipids 2:479 (1967).
15. Hopkins, S.M., G. Sheehan and R.L. Lyman, Biochim. Biophys. Acta 164:272 (1968).
16. Tinoco, J., A. Shannon, P. Miljanich, R.L. Lyman and R. Okey, Anal. Biochem. 3:514 (1962).
17. Snedecor, G.W., "Statistical Methods," Fifth edition., Iowa State College Press, Ames, Iowa, 1956.
18. Blumenstein, J., Can. J. Biochem. 42:1183 (1964).
19. Arvidson, G.A.E., Eur. J. Biochem. 4:478 (1968).
20. Tinoco, J., G. Sheehan, S. Hopkins and R.L. Lyman, Lipids 5:412 (1970).
21. Ansell, G.B., and J.N. Hawthorne, "Phospholipids, Biochim. Biophys. Acta Library," Vol. 3, 1964. p. 147.
22. Pasternak, C.A., and B. Friedrichs, Biochem. J. 119:481 (1970).
23. Bailey, E., C.B. Taylor and W. Bartley, Ibid. 104:1026 (1967).
24. Rytter, D.J., and W.E. Cornatzer, Proc. Soc. Exp. Biol. Med. 134:630 (1970).
25. Beare-Rogers, J.L., Lipids 6:649 (1971).
26. Lyman, R.L., S.M. Hopkins, G. Sheehan and J. Tinoco, Biochim. Biophys. Acta 152:197 (1968).
27. Balint, J.A., D.A. Beeler, D.H. Treble and H.L. Spitzer, J. Lipid Res. 8:486 (1967).
28. Rytter, D., J.E. Miller and W.E. Cornatzer, Biochim. Biophys. Acta 152:418 (1968).
29. Beare-Rogers, J.L., Can. J. Physiol. Pharmacol. 49:171 (1971).

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Effects of Chronic Alcohol Ingestion on the Fatty Acid Composition of the Heart

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ABSTRACT

The effects of alcohol on the total fatty acid composition of the heart have been measured, and two highly significant differences were noted between the alcohol-treated animals and the two control groups. Linoleic acid was elevated, and arachidonic acid was decreased. The increase in linoleate appeared to be a generalized increase within the various lipids analyzed, whereas the decrease in arachidonate was accounted for largely in the phosphatidyl cholines. The ratios of 20:4ω6/18:2 were calculated and compared to data from other researchers. The decrease in this ratio in the hearts from the alcohol-treated animals was similar to the data from the liver of animals treated with various agents which produce a fatty liver. Various mechanisms that could cause such changes in these two essential fatty acids are discussed, and it was concluded that a combination of effects could cause these changes in linoleate and arachidonate. These effects include: (a) increased mobilization of linoleate from adipose tissue; (b) decreased β-oxidation of linoleate; and (c) inhibition of the elongation-desaturation system which converts linoleate to arachidonate.

INTRODUCTION

Alterations have been noted in myocardial morphology and related to alcoholism by several workers (1-5). Wendt et al. (6) have observed the release of certain myocardial enzymes, i.e., malate dehydrogenase and NADP-specific isocitrate dehydrogenase, in both normal and alcoholic men 30 min after ingesting 6 oz of vodka. These data suggested that alcohol may alter membrane permeability and affect the activity of metabolic pathways. Song and Rubin (7) have shown that chronic alcohol ingestion leads to increased serum creatine phosphokinase activity and to ultrastructural changes in skeletal muscle. They also suggest that these affects may be present in alcoholic cardiomyopathy. In addition, other workers (8-10) have associated changes in fatty acid composition with membrane stability. Therefore, since alcohol has been associated with membrane stability or permeability, or both, this study was undertaken to determine what effects, if any, chronic alcohol ingestion has on the myocardial fatty acid composition.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 110-145 g were matched according to weight at the beginning of the experiments and put into groups of three. The diets were closely controlled and consisted of Purina Lab Chow (3.7 cal./g) and administered according to Fallon et al. (11). One rat in each group of three received 25% (v/v) ethanol as the only source of drinking water; the second animal received an isocaloric amount of glucose in its drinking water, and the third animal received just water to drink. The third animal was kept isocaloric with the other two by increasing the amount of chow. The animals were maintained on these diets for ca. 1 month, at which time they were sacrificed by decapitation.

The heart from each rat was perfused with ice cold isotonic saline immediately after the animal was sacrificed. The hearts were weighed, homogenized in 0.25 M sucrose and extracted with methanol-chloroform 2:1 v/v containing 0.02% butylated hydroxytoluene (BHT) according to the procedure of Bligh and Dyer (12). The fatty acid methyl esters were prepared from the total heart lipid extract by the procedure of Metcalf et al. (13). The gas chromatographic separations of the methyl esters were obtained on an EGSS-Y column as described previously (14). Initial gas chromatography of the total heart fatty acids revealed only trace amounts of arachidic acid; therefore 20:0 was added to the total lipid extract prior to the preparation of the methyl esters as an internal standard. This procedure allowed the measurement of the dimethyl acetals produced from the vinyl ethers. Since the antioxident BHT chromatographed similarly to methyl myristate, data were not obtained for this fatty acid.

The lipids were separated by thin layer chromatography (TLC) on Silica Gel H into the phosphatidyl cholines, the phosphatidyl ethanolamines, the cardiolipins and the neutral lipids. The solvent system was chloroform-methanol-ammonia-water 60:30:2.5:2.5. The lipids were located by spraying with 0.01% 2,7-dichlorofluorescein in methanol and using UV light. The internal standard was then added

TABLE I

Comparison of Total Heart Fatty Acid Compositions from Ethanol-Treated Rats with those from Glucose and Chow Controls[a]

Fatty acid	Mol %/g wet wt			Statistical comparison	
	Ethanol	Glucose	Chow	Ethanol vs. glucose	Ethanol vs. chow
DMA[a]	1.07 ± .08	1.27 ± .08	1.30 ± .15	N.S.[c]	N.S.
16:0	11.29 ± .42	12.51 ± .48	11.63 ± .39	N.S.	N.S.
16:1	1.10 ± .11	1.26 ± .11	.82 ± .06	N.S.	N.S.
DMA	.73 ± .13	.32 ± .03	.34 ± .02	.02> P> .01	N.S.
DMA	.88 ± .06	.68 ± .04	.86 ± .03	.01> P> .001	N.S.
18:0	18.25 ± .38	19.29 ± .44	19.73 ± .36	N.S.	N.S.
18:1	13.39 ± .61	14.52 ± .49	12.16 ± .19	N.S.	N.S.
18:2	25.67 ± .68	18.14 ± .43	20.42 ± .52	P< .001	P< .001
20:3ω6	.64 ± .05	.54 ± .03	.46 ± .03	N.S.	N.S.
20:4ω6	12.61 ± .38	16.62 ± .38	15.66 ± .44	P< .001	P< .001
20:5ω3	.86 ± .07	.61 ± .07	.98 ± .09	.02> P> .01	N.S.
22:4ω6	.44 ± .04	.44 ± .02	.34 ± .02	N.S.	N.S.
22:5ω6	.47 ± .06	.48 ± .06	.59 ± .05	N.S.	N.S.
22:5ω3	2.06 ± .10	2.19 ± .11	2.50 ± .08	N.S.	.05> P> .02
22:6ω3	10.75 ± .57	12.25 ± .38	12.34 ± .51	.05> P> .02	N.S.

[a]All values are the means ± standard error from the analysis of 21-26 animals except the data for the chow control which represents only seven animals.

[b]DMA = dimethyl acetal.

[c]Not significant.

directly to the different bands, the bands scraped from the plates and the methyl esters prepared directly from the scrapings.

RESULTS AND DISCUSSION

The effects of alcohol on the total fatty acid composition of the heart have been measured, and two highly significant differences were noted between the alcohol-treated animals and the two control groups. These differences, shown in Table I, were that linoleic acid was elevated in the alcohol-treated animals compared to either the chow or glucose control groups and that arachidonic acid was decreased in the alcohol-treated animals compared to either of the two control groups. These changes were highly significant and had P values <0.001. Other changes were noted to be statistically different, but the level of significance was not as great as that for 18:2 or 20:4ω6. When comparing the alcohol group with the glucose control group, two of the dimethyl acetals and 20:5ω3 were elevated while 22:6ω3 was decreased. The decrease in 22:6ω3 was statistically different, but the level of significance was borderline with P lying between 0.05 and 0.02. When comparing the alcohol group with the chow control group, only one fatty acid other than 18:2 and 20:4ω6 was found statistically different. This was a decrease in 22:5ω3 in the alcohol group; however the level of significance was again borderline with P lying between 0.05 and 0.02. One other difference to be found in Table I was that the sum of all the polyenoic fatty acids, except 18:2 was decreased ca. 13% in the alcohol group compared to either of the two control groups.

In an effort to determine if any specific lipid contained either high percentages of 18:2 or low percentages of 20:4ω6, a single representative sample was taken from the alcohol group and from the glucose group, and the lipids were separated into the phosphatidyl cholines, the phosphatidyl ethanolamines, the cardiolipins and the neutral lipids. The methyl esters were prepared, and the various fatty acids were quantitated. The changes of 18:2 in the heart were not localized in any one particular lipid, because the phosphatidyl cholines, the phosphatidyl ethanolamines, the cardiolipins and the neutral lipids all exhibited similar increases in this fatty acid. In addition, arachidonate was largely accounted for by the decrease observed in the phosphatidyl cholines.

There is strong evidence that ethanol ingestion increases fatty acid mobilization from adipose tissue (17-21). In addition, several workers have shown that there is a very close similarity between the fatty acid composition of adipose tissue triglycerides and liver triglycerides after alcohol administration (17,18,22,23). The changes that might be expected from such mobilization, as increases in palmitic, oleic and linoleic acids and decreases in stearic and arachidonic acids, were not observed in either the total lipids or the

TABLE II

Arachidonate-Linoleate Ratios in Various Lipids from Animals Treated by Different Fatty Liver-Producing Conditions

A.

Source of lipid	Treatment of animal, ratios		
	Chow	Glucose	Ethanol
Total heart fatty acids	0.767	0.916	0.491
Total heart PC[a]	—	1.22	0.169
Total heart PE[b]	—	2.96	1.21
Total heart Cardiolipin	—	0.112	0.045
Total heart Neutral lipids	—	0.212	0.066

B.

	Ad libitum (chow)	Sucrose	Ethanol (15)
Total liver mitochondrial lipid	2.0	2.2	1.6
Total liver mitochondrial PC	2.7	3.7	1.9
Total liver mitochondrial PE	4.3	6.9	6.4

C.

	Saline	CCl_4 + Asp[c]	CCl_4 (30)
Total liver fatty acids	1.62	1.46	0.93

D.

	Ad libitum (chow)	Pair fed	Ethionine (32)
Total liver phospholipid	1.98	1.54	0.74
Total liver PC	2.88	1.44	0.59
Total liver PE	4.07	1.74	1.41
Total liver triglyceride	0.138	0.082	0.090

[a]PC = phosphatidyl choline.
[b]PE = phosphatidyl ethanolamine.
[c]Asp = L-asparagine.

phospholipids except for the linoleate and arachidonate changes. The neutral lipids, however, did exhibit all these changes except for oleic acid which was decreased by ca. 42%. Evans and coworkers (24-26) have demonstrated an inverse relationship between the uptake of fatty acids by myocardial tissue and the chain length of saturated fatty acids. They have also shown that the monenoic acids were extracted in preference to saturated acids of the same chain length. Of the 18-carbon fatty acids, linoleic acid was extracted less readily than oleic but in preference to stearic acid. Little data are present concerning the relative rates of oxidation of saturated vs. unsaturated fatty acids; however, since there is selective uptaken, there may well be a selective utilization of these fatty acids. If this were true, a selective utilization of saturated and monoenoic acids might offset the increased availability due to mobilization with the net result being an increase in the content of linoleic acid. The decrease in arachidonate and the other polyenes might simply be due to the smaller amounts of these acids available from the circulation. Furthermore an elevation of 18:2, which cannot be synthesized by the rat and is stored in the adipose tissue, indicates that mobilization of fatty acids could very well explain these observations. In addition, the recent data of Parker and Reitz (unpublished) suggested that there may be a decrease in the β-oxidation of linoleate compared to palmitate. This would aid in increasing the linoleate concentration in heart tissue. This fact would also support the concept of selective utilization of fatty acids by heart tissue, and thus help to explain why palmitic and oleic acids did not accumulate in the hearts from the alcohol-treated animals. Studies are in progress to more clearly determine the effects of alcohol on the oxidation of different fatty acids.

French and Morin (15) called attention to the arachidonate-linoleate ratio when they reported that alcohol ingestion caused a decrease in this ratio in the total liver lipids as well as in

the phosphatidyl cholines and phosphatidyl ethanolamines. In addition, similar changes have been documented in the inner mitochondrial and microsomal membranes (16). Other researchers have reported fatty acid analyses of various liver lipid fractions from animals maintained on different diets which produce fatty liver. The ratios of 20:4ω6/18:2 have been calculated from their data, and these figures are presented in Table II for comparison to the data reported herein. The important thing this table showed was that all of the different conditions (CCl_4-, ethanol or ethionine treatment) produced a decrease in the 20:4ω6/18:2 ratio. It should be reemphasized that all of the conditions (CCl_4-, ethanol- or ethionine-treat-fatty liver but each by different mechanisms. The data of French and Morin (15) concerning the effects of alcohol on this ratio in the liver were quite similar to the effects noted for the heart lipids as calculated from Table I. The glucose-treated animals had the highest ratio with the chow control being slightly lower, and the alcohol-treated animals had the lowest ratio.

DePury and Collins (8) have postulated that a decrease in arachidonate may account for the mitochondrial fragility noted in essential fatty acid (EFA) deficiency, because phospholipids deficient in 20:4ω6 did not bind as tightly to structural membrane protein as did phospholipids rich in this fatty acid. Hayashida and Portman (9) have also associated alterations in fatty acid composition due to EFA deficiency with membrane stability. Interestingly, the calculated ratio of 20:4ω6/18:2 from the data of Peluffo et al. (27) increased in the heart from 2.13 in the control to 3.09 in the EFA-deficient animal, but decreased in the liver from 2.20 in the control to 0.38 in the EFA-deficient animal. This ratio in the heart of EFA animals (27) was the reverse of what has been observed in the hearts and livers from alcohol-, CCl_4- and ethionine-treated animals (Table II); however this ratio in the liver of the EFA animals was similar in that it was decreased. One very important fact which must be taken into consideration is that the total fatty acid pattern changed dramatically in EFA-deficient animals, whereas the overall general pattern of fatty acids in the alcohol-treated animals did not change too much. Thus it would surely be naive to suggest that such small alterations in fatty acid composition would explain the membrane damage which has been documented in alcoholic cardiomyopathy by Burch and coworkers (1-3).

Shaw et al. (16) suggested that the decreased 20:4ω6/18:2 rato in liver mitochondria from animals treated with alcohol might be due to a decrease in the chain elongation-desaturation system. Since heart will readily form arachidonate from linoleate, as demonstrated in beef heart mitochondria by Dahlene and Porter (28) and in heart tissue culture by Haggerty et al. (29), this suggestion may also be applicable to the effects of alcohol on the 20:4ω6/18:2 ratio in heart lipids. Thus a combination of effects may lead to the fatty acid compositional changes observed in the heart tissue of alcohol-treated rats: (a) increased mobilization of linoleate from adipose tissue; (b) decreased β-oxidation of linoleate in the heart; and (c) inhibition of elongation-desaturation system which converts the linoleate to arachidonate. The latter would also account for the decrease noted in arachidonate levels.

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REFERENCES

1. Ferrans, V.J., R.G. Hibbs, D.C. Weilbaecher, W.C. Black, J.J. Walsh and G.E. Burch, Amer. Heart J. 69:748 (1965).
2. Hibbs, R.G., V.J. Ferrans, W.C. Black, D.C. Weilbaecher, J.J. Walsh and G.E.Burch, Ibid. 69:766 (1965).
3. Burch, G.E., and N.P. DePasquale, in "Biochemical and Clinical Aspects of Alcohol Metabolism," Edited by V.M. Sardesai, C.C. Thomas, Springfield, Ill., 1969, p. 273.
4. Alexander, C.S., Amer. J. Med. 41:229 (1966).
5. Alexander, C.S., Brit. Heart J. 29:200 (1967).
6. Wendt, V.E., C. Wu, R. Balcon, G. Doty and R.J. Bing, Amer. J. Cardiol. 15:175 (1965).
7. Song, S.K., and E. Rubin, Science 175:329 (1972).
8. DePury, G.G., and F.D. Collins, Chem. Phys. Lipids 1:1 (1966).
9. Hayashida, T., and O.W. Portman, J. Nutr. 81:103 (1963).
10. Hølmer, G., and B. Tronier, Lipids 7:534 (1972).
11. Fallon, H.J., P.M. Gertman and E.L. Kemp, Biochim. Biophys. Acta 187:94 (1969).
12. Bligh, E.G., and W.J. Dyer, Can. J. Biochem. Physiol. 37:911 (1959).
13. Metcalf, L.C., A.A. Schmitz and J.R. Pelka, Anal. Chem. 38:514 (1966).
14. Reitz, R.C., Biochim. Biophys. Acta 260:654 (1972).
15. French, S.W., and R.J. Morin, in "Biochemical and Clinical Aspects of Alcohol Metabolism," Edited by V.M. Sardesai, C.C. Thomas, Springfield, Ill., 1969, p. 123.
16. Shaw, G.P., S.W. French, T.J. Ihrig, T.T. Tonaka and R.J. Morin, Lab. Invest. 24:447 (abstr.) (1971).
17. Horning, M.G., E.A. Williams, H.M. Maling and B.B. Brodie, Biochem. Biophys. Res. Comm. 3:635 (1960).

18. Brodie, B.B., W.M. Butler, Jr., M.G. Horning, R.P. Maickel and H.M. Maling, Amer. J. Clin. Nutr. 9:432 (1961).
19. Scheig, R., and K.J. Isselbacker, J. Lipid Res. 6:269 (1965).
20. Mallov, S., Quart. J. Stud. Alcohol 22:250 (1961).
21. Schapiro, R.H., R.L. Scheig, G.D. Drummey, J.H. Mendelson and K.J. Isselbacker, New Eng. J. Med. 272:610 (1965).
22. Kessler, J.I., and S. Yalovsky-Mishkin, J. Lipid Res. 7:772 (1966).
23. Lieber, G.S., N. Spritz and L.M. DeCarli, J. Clin. Invest. 45:51 (1966).
24. Evans, J.R., Circulation Res. 15 (Suppl. II): 96 (1964).
25. Evans, J.R., C.H. Hollenberg and A. Angel, Fed. Proc. 22:376 (1963).
26. Evans, J.R., Can. J. Biochem. 42:955 (1964).
27. Peluffo, R.O., R.R. Brenner and O. Mercuri, J. Nutr. 81:110 (1963).
28. Dahlene, J.V., and J.W. Porter, Arch. Biochem. Biophys. 127:207 (1968).
29. Haggerty, D.F., L.E. Gerschenson, I. Harary and J.F. Mead, Biochem. Biophys. Res. Comm. 21:568 (1965).
30. Alexander, N.M., R. Scheig and G. Klatskin, Biochem. Pharm. 16:1091 (1967).
31. Sgoutas, D.S., Metabolism. 16:382 (1967).
32. Lyman, R.L., M.A. Fosmire, C. Giotas and P. Miljanich, J. Nutr. 94:74 (1968).

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Biosynthesis of Liver Glycerolipids from Normal and Essential Fatty Acid-Deficient Rats: ^{3}H-Glycerol and 1-^{14}C Linoleic Acid Incorporation

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ABSTRACT

Normal and essential fatty acid (EFA)-deficient rats were injected via the portal vein with a labeled solution containing ^{3}H-glycerol and 1-^{14}C-linoleic acid during a 1 min period. Livers were immediately frozen, pulverized, and the lipids extracted and fractioned by thin layer chromatography. The incorporation of ^{3}H-glycerol and 1-^{14}C-linoleic acid into the different lipid fractions was measured, and the per cent distribution and specific radioactivity determined. A parallel increase was found between the specific activity and the amount of ^{3}H-glycerol incorporated into 1,2-diglycerides, triglycerides, lecithin and cephalin from EFA-deficient and normal rats. Since the amount of glycerol in each fraction studied was quite similar in both groups of rats, these findings can explain the increase in the specific activity observed in the EFA-deficient rats. Nevertheless these facts do not necessarily imply an increased turnover rate of these molecules, since we do not know the specific radioactivity of the 1,2-diacylglycerol precursors. A remarkable increase in the specific radioactivity of the ^{14}C-linoleic acid incorporated into lipid fractions from EFA-deficient rats compared with control rats was observed. While the amount of 1-^{14}C-linoleic acid incorporated into neutral lipids was similar in both groups of rats, a statistically significant increase in the amount of the label incorporated into phospholipids from EFA-deficient rats was observed. These facts suggest an increased turnover rate of the radiolinoleic acid into phospholipid molecules from EFA-deficient rats via deacylation-reacylation pathway.

INTRODUCTION

Previous investigations on essential fatty acid (EFA)-deficient rats showed an increased turnover of ^{32}P in the phospholipids in muscle (1). This finding was extended by Collins (2) to the phospholipids from liver. Stein and Stein (3) found that the specific activity of the labeled linoleic acid incorporated in vitro into phospholipids from heart and diaphragm of EFA-deficient rats was higher than in nondeficient control, indicating an increased turnover of this acid in the phospholipid molecule. In a recent report (4) it was indicated that in the EFA-deficient rats, 1-^{14}C-linoleic acid incorporation was actually directed to the phospholipid fractions of rat liver and not to the triacylglycerol fractions as was observed in the normal rats.

This study deals with an attempt to elucidate the tendency of linoleic acid to be incorporated predominantly into the phospholipid fractions of liver of EFA-deficient rats.

EXPERIMENTAL PROCEDURES

Materials

1-^{14}C-linoleic acid (52.9 mC/mmol) was purchased from The Radiochemical Centre (Amersham, England). It was 98% radiochemically pure and had 2% *cis-trans* unsaturated acid. Glycerol 2-T (*n*) (420 mC/mmol) was also purchased from The Radiochemical Centre.

Animals

Weanling Wistar rats were maintained during 12 weeks on a semisynthetic fat-free diet (5). The diet of the control animals was supplemented with 6% sunflower oil.

Preparation of Injection Solution

The 1-^{14}C-linoleic acid solution was prepared by the method of Elovson (6) and after standing overnight at room temperature under N_2 with magnetic stirring, ^{3}H-glycerol was added; 0.2 ml contained 10 μC of 1-^{14}C-linoleic acid and 50 μC of ^{3}H-glycerol.

Injection of Labeled Solution

Labeled solution was injected intraportally in 0.2 ml portions per animal as described previously (7). Livers were frozen and removed 5 sec after injection.

[1] Member of the Carrera del Investigador Científico of the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

TABLE I

Per Cent of Total Radioactivity Recovered from ^{3}H-Glycerol and ^{14}C-Linoleic Acid Incorporated into Lipid Fractions

Experimental group	Per cent distribution of radioactivity							
	1,2-Diacylglycerol		Triacylglycerol		3-*sn*-Phosphoryl-choline		3-*sn*-Phosphoryl-ethanolamine	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Normal[a,b] (7)	52.9 ±[c] 14.5	24.5 ± 5.0	31.5 ± 8.8	27.1 ± 4.3	7.8 ± 0.8	22.5 ± 2.5	4.8 ± 1.8	7.6 ± 2.0
EFA-[d] deficient (7)	60.3 ± 20.0	17.5 ± 4.9	28.4 ± 3.8	15.5 ± 1.0	8.4 ± 2.3	48.0 ± 3.3	7.0 ± 1.6	11.8 ± 1.0
	NS[e]	P<0.05	NS	P<0.001	NS	P<0.001	NS	P<0.01

[a]Numbers in parentheses indicate the number of animals in each group.

[b]Probability (P) values are related to normal animals.

[c]Data are the means ± standard deviations of the means.

[d]EFA = essential fatty acids.

[e]NS = not significant.

Separation of Lipids

Phospholipids and neutral lipids were separated by thin layer chromatography (TLC) (7), transesterified with methanolic HCl (8), extracted with light petroleum, evaporated and assayed for radioactivity.

Phosphatidylcholine and phosphatidylethanolamine, separated by TLC, were extracted from the silica gel using the two phase system of Arvidson (9). 1,2-Diglyceride and triacylglycerol were extracted from the silica gel using chloroform-methanol 2:1 v/v.

Determination of Specific Radioactivity

The amount of each fraction, separated as described above, was determined by a glycerol analysis according to Snyder and Stephens (10).

The linoleic acid content of each sample was determined by gas liquid chromatography of the fatty acid methyl esters by the use of a suitable internal standard (methyl-11-eicosaenoate). Gas chromatography analyses were carried out on a Pye apparatus with argon ionization detector in a 6 ft column packed with 15% diethyleneglycosuccinate on Chromosorb W 60-80 mesh at 180 C.

Radioactivity measurements were performed in a Packard Tri-Carb Scintillation spectrometer as described earlier (11).

RESULTS AND DISCUSSION

In mammalian liver, *sn*-glycerol-3-phosphate is acylated by acyl-CoA esters to phosphatidic acid. Dephosphorylation of phosphatidic acid produces 1,2-diacylglycerol which is the immediate precursor used for de novo synthesis of triacylglycerol, phosphatidylcholines and phosphatidylethanolamines (12). It is evident that the incorporation of ^{3}H-glycerol is a good marker for de novo biosynthesis of lipid fractions (13).

Table I summarizes the relative distribution of the total radioactivity expressed as percentage of ^{3}H-glycerol and ^{14}C-linoleic acid incorporated into the principal lipid fractions.

As is clearly shown, no significant differences were observed in the distribution of ^{3}H counts in 1,2-diacylglycerol, triacylglycerol, 3-*sn*-phosphatidylcholine and 3-*sn*-phosphatidylethanolamine from normal rats compared with EFA-deficient rats. In contrast, the tendency of 1-^{14}C-linoleic acid to be incorporated predominantly into phospholipids was greater in EFA-deficient rats than in dietary controls.

The latter findings suggest that the incorporation of 1-^{14}C-linoleic acid could be done through the deacylation-reacylation cycle (14) with monoacylphosphoglycerides as intermediates rather than by de novo biosynthesis (12).

Table II summarizes the capacity of the liver to incorporate the injected ^{3}H-glycerol and ^{14}C-linoleic acid into lipid fraction. The total radioactivity of ^{3}H-glycerol incorporated per gram of liver is about twice as high in all the lipid fractions of EFA-deficient rats compared with normal rats.

A similar incorporation of 1-^{14}C-linoleic acid into the 1,2-diacylglycerol fraction from both groups of rats was observed. In contrast, in the triacylglycerol fraction the amount of labeled acid incorporated was smaller in the EFA-deficient group. This result agrees with Table I.

This fact could be due to a lack of linoleic acid in the intracellular acyl-CoA pool of the EFA-deficient animals, since the fatty acid pattern at the 3 position of triacylglycerol may be a reflection of the composition of the

endogenous acyl-CoA pool (15,16).

On the other hand, significantly more labeled linoleic acid was recovered in the phospholipid fractions of EFA-deficient rats when compared to controls.

There are at least three possibilities to explain these findings: (a) An elevated activity of the enzyme acyl-CoA lysophosphatidetransferase, as demonstrated in vitro by Lands et al. (17) in EFA-deficient rats. (b) Collins (2) attributed the increased ^{32}P turnover in phospholipids from EFA-deficient rat liver to the lack of a more stable lecithin fraction containing arachidonic acid. The lack of arachidonic acid could promote the hydrolysis by phospholipase A (18) which would result in an increased formation of lysolecithin which has been shown to be an avid polyunsaturated fatty acid acceptor, in the pathway of lecithin synthesis (18-20). (c) A high specific activity of the 1-^{14}C-linoleic acid incorporated by this pathway is a third possibility, since there would be a lack of linoleic acid in the acyl-CoA pool.

Table III shows the specific radioactivity of ^{3}H-glycerol and 1-^{14}C-linoleic acid in each lipid fraction. These values could be modified by at least three factors: (a) the steps preceding its incorporation; (b) the amount of radioactivity incorporated; (c) the dilution of the labeled compound into the lipid fraction in which is already incorporated.

As is clearly shown (Table III) the specific radioactivity of ^{3}H-glycerol is about twice as high in all the lipid fractions studied from EFA-deficient rats compared with normal rats.

Since similar pool sizes of each lipid fraction obtained from both groups of rats referred to their glycerol content, this in turn could account for a rather similar dilution of ^{3}H-glycerol incorporated into these liver lipids fractions. Furthermore the amount of ^{3}H radioactivity incorporated into these fractions was twice as great in the EFA-deficient rats as in the control group (Table II). These findings could explain the high specific radioactivity, about twice as high, found in the ^{3}H-glycerol incorporated into lipid fractions of EFA-deficient rats when compared with normal rats. Nevertheless it is necessary to emphasize that the increase in the amount of ^{3}H radioactivity incorporated into 1,2-diacylglycerol does not necessarily imply an increase in the actual amount of glycerol incorporated, since we do not know the dilution of the injected ^{3}H-glycerol before its incorporation.

On the other hand, the increase in the specific radioactivity of ^{3}H-glycerol incorporated into triacylglycerol, lecithin and cephalin fractions from EFA-deficient rats could be

TABLE II

Amount of ^{3}H-Glycerol and ^{14}C-Linoleic Acid Incorporated into Lipid Fractions

Experimental group	dpm/g Wet tissue							
	1,2-Diacylglycerol		Triacylglycerol		3-*sn*-Phosphorylcholine		3-*sn*-Phosphorylethanolamine	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Normal[a,b] (7)	250,899±[c]	56,529±	149,401±	62,528±	36,994±	51,914±	22,765±	17,535±
	68,917	11,536	41,737	9,921	3,974	5,768	8,537	4,614
EFA-deficient[d] (7)	584,153±	53,237±	275,123±	47,153±	81,374±	146,023±	67,812±	35,897±
	193,749	14,906	36,812	3,042	22,281	10,039	15,499	3,042
	P<0.01	NS[e]	P<0.001	P<0.01	P<0.001	P<0.001	P<0.001	P<0.001

[a]Numbers in parentheses indicate the number of animals in each group.
[b]Probability (P) values are related to normal animals.
[c]Values are the means ± standard deviations of the means.
[d]EFA = essential fatty acids.
[e]NS = not significant.

TABLE III

Specific Radioactivity of ^{3}H-Glycerol and ^{14}C-Linoleic Acid Incorporated into Lipid Fractions

Experimental group	dpm/μmol							
	1,2-Diacylglycerol		Triacylglycerol		3-*sn*-Phosphorylcholine		3-*sn*-Phosphorylethanolamine	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Normal[a,b] (7)	30.0x[c]	271.0x[d]	2.6x	21.0x	1.1x	6.2x	0.6x	6.0x
	10^4	10^4 ±	10^4 ±	10^4 ±	10^4 ±	10^4 ±	10^4 ±	10^4 ±
		50.0x	0.7x	2.0x	0.5x	2.6x	0.1x	2.0x
		10^4	10^4	10^4	10^4	10^4	10^4	10^4
EFA-deficient[e] (7)	90.0x[c]	2037.0x	4.5x	217.0x	2.6x	142.0x	1.0x	138.0x
	10^4	10^4 ±	10^4 ±	10^4 ±	10^4 ±	10^4 ±	10^4 ±	10^4 ±
		60.0x	0.6x	60.0x	0.8x	27.0x	0.2x	40.0x
		10^4	10^4	10^4	10^4	10^4	10^4	10^4
		P<0.001	P<0.01	P<0.001	P<0.01	P<0.001	P<0.01	P<0.001

[a]Numbers in parentheses indicate the number of animals in each group.
[b]Probability (P) values are related to normal animals.
[c]Pooled samples.
[d]Values are the mean ± standard deviations of the means.
[e]EFA = essential fatty acid.

explained by the increase in the specific radioactivity of 1,2-diacylglycerol common substrate in the metabolic pathway of these molecules.

The specific radioactivity of 1-^{14}C-linoleic acid was markedly increased in the deficient animals (Table III). The specific radioactivity of 1-^{14}C-linoleic acid incorporated into the neutral lipids was ca. 10-fold higher in the EFA-deficient rats than in control rats.

Since the amount of 1-^{14}C radioactivity incorporated in 1,2-diacylglycerol and triacylglycerol fractions was similar in both groups of rats (Table II), and the decrease in the content of linoleic acid in both fractions was ca. 10 times smaller in the EFA-deficient group, as was demonstrated by gas liquid chromatography, these facts could be enough to explain the increase in the specific radioactivity of 1-^{14}C-linoleic acid observed in neutral lipids of EFA-deficient rats.

If we assumed, as seems likely, that the specific radioactivity of 1-^{14}C-linoleic acid in the endogenous acyl-CoA pool could be higher in the EFA-deficient rats than in the controls, these findings would point to a decreased turnover rate of the linoleic acid in the neutral lipid fractions. On the other hand, the increase in the specific radioactivity of 1-^{14}C-linoleic acid observed in the phospholipid fraction of EFA-deficient rats as compared with controls, cannot be explained only by the decrease in the linoleic acid content of this fraction from EFA-deficient rats but also by the increase in the 1-^{14}C-linoleic acid incorporated (Table II).

Therefore these findings point to an increased turnover rate of the 1-^{14}C-linoleic acid of the phospholipid molecules of essential fatty acid deficient rats.

According to these results, we can speculate that the deacylation-reacylation pathway (13) could be responsible for this increased turnover rate.

ACKNOWLEDGMENTS

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REFERENCES

1. Hevesy, G.C., and T. Smedley-Maclean, Biochem. J. 34:903 (1940).
2. Collins, F.D., Biochem. Biophys. Res. Com. 9:289 (1962).
3. Stein, O., and Y. Stein, Biochim. Biophys. Acta 84:621 (1964).
4. Mercuri, O., and M.E. De Tomás, Lipids 6:888 (1971).

5. Peluffo, R.O., R.R. Brenner and O. Mercuri, J. Nutr. 81:110 (1963).
6. Elovson, J., Biochim. Biophys. Acta 106:480 (1965).
7. De Tomás, M.E., and O. Mercuri, Lipids 6:787 (1971).
8. Stoffel, W., F. Chu and E.H. Ahrens, Anal. Chem. 31:307 (1959).
9. Arvidson, G.A.E., Eur. J. Biochem. 4:478 (1968).
10. Snyder, F., and N. Stephens, Biochim. Biophys. Acta 34:244 (1959).
11. Mercuri, O., and M.E. De Tomás, Lipids 6:203 (1971).
12. Kennedy, E.P., Fed. Proc. 16:847 (1957).
13. Lands, W.E.M., J. Biol. Chem. 235:2233 (1960).
14. Åkesson, B., J. Elovson and G. Arvidson, Biochim. Biophys. Acta 210:15 (1970).
15. Slakey, S.P., and W.E.M. Lands, Lipids 3:30 (1968).
16. Åkesson, B., Eur. J. Biochem. 9:463 (1969).
17. Lands, W.E.M., M.L. Blank, L.J. Nutter and O.S. Privett, Lipids 1:244 (1966).
18. Van Den Bosch, H., and L.L.M. Van Deenen, Biochim. Biophys. Acta 106:326 (1965).
19. Stoffel, W., H.G. Schiefer and G.D. Wolf, Hoppe-Seyler's Z. Phys. Chem. 347:102 (1966).
20. Stoffel, W., M.E. De Tomás and H.G. Schiefer, Ibid. 348:882 (1967).

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Identification of 18-Oxo-9,10-Epoxystearic Acid, a Novel Compound in the Cutin of Young Apple Fruits[1]

ABSTRACT

18-Oxo-9,10-epoxystearic acid was identified in the cutin of young apple fruits by hydrogenolysis with $LiAlH_4$, deuterolysis with $LiAlD_4$, CrO_3 oxidation of deuterated product followed by reduction with $LiAlH_4$ and mass spectrometry of the products.

INTRODUCTION

Cutin, the structural component of plant cuticle is a polymer of hydroxy fatty acids (1). Hydrolytic cleavage (2-4) or reductive cleavage (5) of the polymer followed by gas liquid chromatography and mass spectrometry (GLC-MS) was recently used to determine the structure of the mono- and polyhydroxy fatty acids. Recent work in this laboratory revealed that in the very young tissue hitherto unknown monomers might be present. In this communication the discovery of 18-oxo-9,10-epoxystearic acid, a novel monomer in the cutin of young apples, is reported.

[1]Scientific Paper No. 3957, Project 2001, Agricultural Research Center, College of Agriculture, Washington State University, Pullman, Wash.

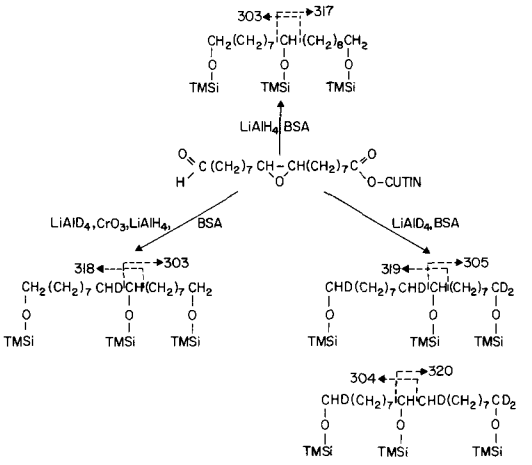

FIG. 1. Expected products from 18-oxo-9,10-epoxystearic acid of cutin by the various treatments and the expected major α-cleavages of the products. BSA = N,O-bis(trimethylsilyl)acetamide.

EXPERIMENTAL PROCEDURES

Young McIntosh apples (4 cm) were collected from the apple orchard of Washington State University. Isolation, hydrogenolysis and deuterolysis (with $LiAlD_4$ > 99%) of cutin were performed as described before (5). The C_{18} triol fraction was isolated by thin layer chromatography (TLC) on Silica Gel G with ethyl ether-hexane-methanol 8:2:1 v/v. This fraction obtained from the deuterolysate was oxidized with CrO_3 (100 mg) in 95% acetic acid at room temperature for ca. 14 hr. After diluting the reaction mixture with water the products were extracted three times with $CHCl_3$. The oxidation product recovered from the chloroform extract was refluxed with $LiAlH_4$ in tetrahydrofuran for ca. 2 hr and the reduction products were extracted with chloroform (5). The C_{18} triol fraction was isolated

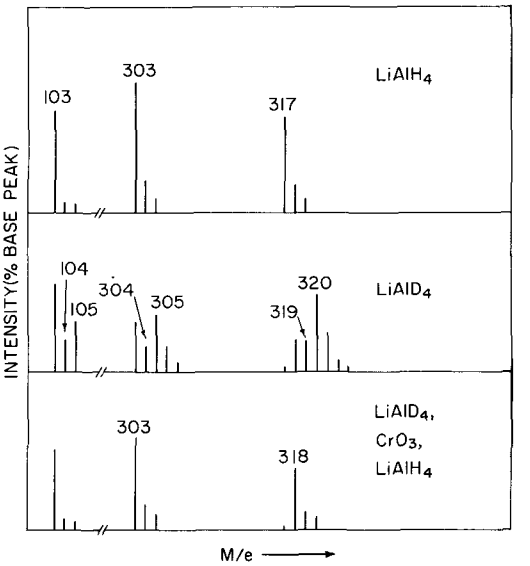

FIG. 2. Partial mass spectra of the C_{18} triols derived by the treatments indicated on the figure.

from this reduction product by TLC as above. The triol was treated with N,O-bis(trimethylsilyl)acetamide (Pierce Chemicals) and the silyl derivative was subjected to GLC-MS (5).

RESULTS AND DISCUSSION

Cutin from mature apples contains 18-hydroxy-9,10-epoxystearic acid and its Δ^{12} unsaturated analog (3,5-7). The C_{18} triol fraction obtained by hydrogenolysis of such cutin was shown to be derived from the above epoxy acid (5). In order to determine whether this component is present in the cutin of immature apple fruits, the C_{18} triol obtained by hydrogenolysis of cutin from young apple fruits was subjected to GLC-MS. The mass spectrum was identical to that of the C_{18} triol derived from cutin of mature apple. The major α-cleavage ions were at m/e 303 and 317. Since hydrogenolysis converts the carboxyl end to primary alcohol and the epoxide into secondary alcohols, the exact structure of this component in the cutin could not be determined from this spectrum. In order to overcome this difficulty, the C_{18} triol derived from the deuterolysate of cutin was analyzed. The mass spectrum of this component showed the same pattern as that observed with the cutin of mature apple. However M^+, M^+-CH_3 and M^+-CH_3-$(CH_3)_3$ SiOH showed the presence of four deuterium atoms rather than the three expected from the 18-hydroxy-9,10-epoxystearic acid. The intensity of ions at m/e 105 and 104 indicated that $(CD_2OSi[CH_3]_3)^+$ and $(CHDOSi[CH_3]_3)^+$ were formed, suggesting that one end of the molecule was derived from an aldehyde function. If 18-oxo-9,10-epoxystearic acid gave rise to the triol fraction, the α-cleavage ions expected would be at m/e 305, 319, 304 and 320 (Fig. 1). These ions were major α-cleavage ions in the spectrum (Fig. 2). The fact that ions at m/e 303 and 318 were present indicate that part of the C_{18} triol was derived from 18-hydroxy-9,10-epoxystearic acid (5). The relative intensities of the various α-cleavage ions observed in the spectrum (Fig. 2) are in agreement with such a structural assignment.

If the four deuterium atoms found in the C_{18} triol were introduced by the reduction of the corresponding dicarboxylic acid, the CrO_3 oxidation product of the triol would contain no deuterium. If an in-chain deuterium were introduced by the reduction of a carbonyl group, this deuterium would also be lost by the mild CrO_3 oxidation, because the in-chain hydroxyl group is oxidized to a ketone by CrO_3. On the other hand, if an in-chain deuterium originated by reduction of an epoxide, it would not be affected by the CrO_3 treatment. If the tetradeuterated C_{18} triol was in fact derived from 18-oxo-9,10-epoxystearic acid of the cutin as shown in Figure 1, mild CrO_3 oxidation of the triol should remove the three deuterium atoms from the terminal carbon atoms leaving only the one deuterium atom in the chain. Thus the two isomeric tetradeutero C_{18} triols should give identical monodeuteroketodicarboxylic acid, which on reduction with $LiAlH_4$ should give the monodeuteriotriol shown in Figure 1. The mass spectrum of the triol obtained in this manner showed M^+, M^+-CH_3 and M^+-CH_3-$(CH_3)_3SiOH$ ions 1 amu higher than those from the C_{18} triol of the cutin hydrogenolysate. The relative intensities of ions at m/e 103, 104 and 105 clearly showed that the deuterium from the terminal carbons had been removed. The major α-cleavage ions were exclusively at m/e 303 and 318 as predicted from the structural assignments (Fig. 1). Thus the in-chain deuterium originated by the reduction of an epoxide. These results clearly show that the cutin of immature apple contained 18-oxo-9,10-epoxystearic acid. This compound does not appear to have been reported to be present in nature. The deuterium labeling method coupled to GLC-MS allows us to identify monomers that contain functional groups such as aldehyde and epoxide, which are highly unsatable to conditions used in the conventional depolymerization techniques.

The biological significance of the occurrence of aldehydes in the cuticular polymer is not clear. The most likely precursor of the 18-oxo-9,10-epoxystearic acid is 18-hydroxy-9,10-epoxystearic acid which is probably derived from ω-hydroxyoleic acid. Oleic acid has been shown to be converted into ω-hydroxyoleic acid in this tissue (7).

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REFERENCES

1. Kolattukudy, P.E., and T.J. Walton, Prog. Chem. Fats and Other Lipids 13:119 (1972).
2. Eglinton, G., and D.H. Hunneman, Phytochem. 7:313 (1968).
3. Croteau, R., and I.S. Fagerson, Ibid. 11:353 (1972).
4. Brieskorn, C.H., and K. Beck, Ibid. 9:1633

(1970).
5. Walton, T.J., and P.E. Kolattukudy, Biochemistry 11:1885 (1972).
6. Brieskorn, C.H., and J. Boss, Fette Seifen Anstrichm. 66:925 (1964).
7. Kolattukudy, P.E., T.J. Walton and R.P.S. Kushwaha, Biochem. Biophys. Res. Comm. 42:739 (1971).

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Prostaglandins in Swine Testes

ABSTRACT

The prostaglandin content of swine testes was investigated. It was found that PGE1, PGE2, PGF1 and PGF2 were present, and the amount of total PGE's and PGF's was determined.

Prostaglandins, a family of closely related endogenous substances, are derived from essential fatty acids. They exert a variety of important biological properties, which have been the subject of extensive investigations.

Prostaglandins have a widespread distribution in the animal body and their occurrence in many tissues and fluids, i.e., lungs, thymus, brain, seminal plasma, male accessory glands, menstrual fluids (1-4), etc., has been reported in the literature for human and mammalian species.

However it is only recently that the occurrence of prostaglandins in testicular tissue of the rat was examined, though quantitative data were not presented.

The present study describes the isolation, identification and quantitative estimation of prostaglandins found in swine testes.

Semifrozen swine testicular tissue (500 g) was cut in slices and homogenized with 95% alcohol (2.5 liters) in a Braun mixer. The homogenate was stirred mechanically for six hr and then centrifuged at 1800 rpm for ca. 45 min. The supernatant was concentrated under reduced pressure (25 C) to the 20th of its volume, acidified to pH 3.5 with 1N hydrochloric acid, and extracted with ether three times with half its volume. The combined ethereal layers was extracted five to six times of half their volume with phosphate buffer pH 8; the organic layers were discarded and the aqueous phases combined, acidified to pH 3.5 and extracted three times with an equal volume of ether. The ethereal phases were combined, washed several times with small portions of water, dried over sodium sulphate and evaporated to dryness under reduced pressure. The remaining paste was dissolved in 60% aqueous ethanol (50 ml) and partitioned three to four times with an equal volume of petroleum ether. The alcoholic layer was then evaporated to a small volume, acidified to pH 3.5 and extracted three times with an equal volume of ether. The combined ethereal extracts were dried over sodium sulphate and evaporated to dryness under reduced pressure.

TABLE I

Prostaglandin Content of Swine Testes

Preparation	Tissue wt, g	Total PGE's, μg/g	Total PGF's, μg/g
I	450	1.5	0.8
II	690	1.7	0.7
III	500	1.2	0.5

The residue was dissolved in benzene (5 ml) and the solution placed on the top of a column of silicic acid (0.5 x 15 cm), prepared from a silicic acid-benzene slurry and washed repeatedly with benzene before use. Elution of the column with benzene (40 ml) afforded the PGB's while the PGE's and PGF's were obtained by further elution of the column with ethyl acetate (50 ml), as indicated by thin layer chromatography (TLC) monitoring of the column using Silica Gel G plates and benzene-dioxan-acetic acid 40:35:2 as solvent system. By this TLC procedure the latter two prostaglandin groups were separated and their total amount estimated as follows: The zones corresponding to the PGE's and PGF's were scraped off separately, transferred to test tubes and extracted with methanol (3 x 5 ml). The meth-

(1970).
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III	500	1.2	0.5

The residue was dissolved in benzene (5 ml) and the solution placed on the top of a column of silicic acid (0.5 x 15 cm), prepared from a silicic acid-benzene slurry and washed repeatedly with benzene before use. Elution of the column with benzene (40 ml) afforded the PGB's while the PGE's and PGF's were obtained by further elution of the column with ethyl acetate (50 ml), as indicated by thin layer chromatography (TLC) monitoring of the column using Silica Gel G plates and benzene-dioxan-acetic acid 40:35:2 as solvent system. By this TLC procedure the latter two prostaglandin groups were separated and their total amount estimated as follows: The zones corresponding to the PGE's and PGF's were scraped off separately, transferred to test tubes and extracted with methanol (3 x 5 ml). The meth-

anolic layers were combined, evaporated to a small volume (3-4 ml) diluted with water (10 ml), acidified to pH 3.5 and extracted three times with an equal volume of ether. The combined ethereal extracts of each prostaglandin group were washed several times with small portions of water, dried over sodium sulphate, and the total PGE content was estimated by their absorption at 278 nm after alkali treatment at room temperature (7), while total PGF content was estimated by their absorption at 300 nm after treatment with concentrated H_2SO_4 at room temperature (5).

Total PGF were also estimated by a spectrofluorimetric method described by Gantt et al. (8). This method gave results lower than that obtained by the first technique. These data are not reported here.

To test the recovery of the method used, an experiment was performed in duplicate exactly as described above, except that 9-^{3}H PGF_{1a} (specific activity 0.1 $\mu C/\mu M$) was added to the original homogenate in alcoholic solution. It was found that 75% and 80% of the radioactivity were recovered from the two experiments, respectively. Table I shows the total amount of PGE's and PGF's estimated for three experimental animals. For the qualitative evaluation of the prostaglandins, mixtures of PGE's were further separated on silver nitrate impregnated Silica Gel G plates with AII as solvent system (9).

Visualization of the spots with 10% alcoholic molybdophosphoric acid indicated the presence of PGE_1, PGE_2, PGF_{1a} and PGF_{2a}.

Small amounts of PGA's and PGB's were also detected, their presence being, most probably, due to a partial dehydration of the PGE's.

In conclusion, swine testes contain the major prostaglandins and in quantities higher than those encountered in other mammalian tissues. In view of the fact that prostaglandins can be synthesized in the testes (6), it remains to be found whether the prostaglandins of the seminal plasma depend in some way upon the presently identified prostaglandins.

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REFERENCES

1. Anggard, E., Biochem. Pharm. 14:1507 (1965).
2. Bergström, S., and B. Samuelsson, Acta Chem. Scand. 17:5282 (1963).
3. Samuelsson, B., Biochim. Biophys. Acta 84:218 (1964).
4. Hamberg, M., and B. Samuelsson, J. Biol. Chem. 241:257 (1966).
5. Bergström, S., and J. Sjöval, Acta Chem. Scand. 14:1693 (1960); Ibid. 14:1701 (1960).
6. Carpenter, M.P., and B. Wisman, Fed. Proc. 29:248 (1970).
7. Bygderman, M., and B. Samuelsson, Clin. Chim. Acta 10:566 (1964).
8. Gantt, C.L., L.R. Kizlaitis, D.R. Thomas and I.G. Greslin, Anal. Chem. 40:2190 (1968).
9. Green, K., and B. Samuelsson, J. Lipid Res. 5:117 (1964).

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LETTER TO THE EDITOR

Positional Isomers of *cis* and *trans* Monoenoic Fatty Acids from Ox (Steer) Perinephric Fat

Sir: We have found that the monoenoic fatty acids (FA) in bovine milk fat contain a large variety of positional and geometric isomers (Hay and Morrison, Biochim. Biophys. Acta 202:237 [1970]). The preponderance of certain isomers aroused speculation as to their biochemical origins and led us to conclude that several of the FA were formed by desaturase and α- and β-oxidation mechanisms operating within the mammary gland. The possibility was overlooked that these transformations may also have occurred at other sites, such as liver, and the products transferred to the mammary gland via the circulatory system. The study of ox (steer) perinephric fat presented here indicates that the monoenoic FA positional isomers found in milk fat are not confined solely to mammary tissue.

Samples of steer perinephric fat were cut into thin strips and extracted with chloroform-methanol 2:1 v/v. The total lipids were purified by the Folch procedure (Folch et al., J. Biol. Chem. 226:497 [1957]) and transesterified with methanolic KOH (Christopherson and Glass, J. Dairy Sci. 52:1289 [1969]). Pure 14:1, 16:1, 17:1 and 18:1 FAMe were isolated as described previously by Hay and Morrison. Gas liquid chromatographic analysis of total FAMe from the steer perinephric fat showed that the proportions of monoenoic FA were 14:1 = 0.9%, 16:1 = 1.3%, 17:1 = 0.4% and 18:1 = 18.9%. The *trans* content of the individual monoenoic FA, determined by IR spectrophotometry and by silver ion thin layer chromatography, were 14:1 = 8.5%, 16:1 = 23.5%, 17:1 = 1.0% and 18:1 = 19.1%.

The *cis* and *trans* positional isomers (Table I) closely resemble those found in bovine milk fat, and theories (Hay and Morrison) pertaining to the origin of these isomers in bovine milk fat can be applied equally well to perinephric fat. Thus the *trans* 18:1 isomers, because of their compositional similarity to the *trans* 18:1 isomers from rumen bacterial lipids, are probably derived from the rumen (Katz and Keeney, J. Dairy Sci. 49:962 [1966]). The 9-*cis* isomers are probably formed from their saturated counterparts by desaturation, the 8-*cis* isomer of 17:1 being formed from 9-*cis*-18:1 by α-oxidation and the *trans* 16:1, 7-*cis*-16:1 and 7-*cis*-14:1 formed from *trans* 18:1, 9-*cis*-18:1 and 9-*cis*-16:1, respectively, by β-oxidation.

TABLE I

Distribution of Double Bonds in *cis* and *trans* Monenoic Fatty Acids in Steer Perinephric fat[a]

Position of double bond	*cis* Isomers				*trans* Isomers	
	14:1	16:1	17:1	18:1	16:1	18:1
5	3.2					
6	3.2	6.6	3.2		4.1	
7	12.2	28.0	6.3	0.5	3.3	1.0
8	5.4	Trace	34.0	1.1	9.2	2.1
9	76.0	58.8	56.5	85.4	43.2	5.0
10		Trace		2.2	6.7	11.9
11		1.2		8.6	9.5	46.9
12		5.3		2.2	11.9	6.0
13					9.5	6.6
14					1.0	7.4
15					1.6	5.5
16						7.6

[a]Values expressed in wt %. Trace = less than 0.1%.

Since these results were obtained with steer perinephric fat, it is evident that the oxidative and desaturase mechanisms must occur wholly or partially outside the mammary gland, contrary to our previous conclusions. Desaturation (Gellerhorn and Benjamin, Biochim. Biophys. Acta 84:167 [1966]; Kellerman and Jollow, Biochim. Biophys. Acta 84:478 [1964]; and Thompson and Allen, J. Animal Sci. 29:127 [1969]) and β-oxidation (Milstein and Driscoll, J. Biol. Chem. 234:19 [1959]) have been observed in adipose tissue and liver (Chang and Holman, Biochim. Biophys. Acta 280:17 [1972]), and the mechanisms which we have proposed to account for the isomeric monoenoic FA in milk (Hay and Morrison) and perinephric fats may occur in the liver, adipose tissue or mammary gland, which are all active sites of lipid metabolism.

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Erratum

In "Presence of *trans*-6-Hexadecenoic Acid in the White Jellyfish *Aurelia aurita* Lamarck and in a Caribbean Gorgonian" (*Lipids* 7:624 [1972]), there is an error in Table I. The fatty acid listed as 20:1ω6 (column 1, line 18) should be 20:4ω6.

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Purification of Sitosterol[1]

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ABSTRACT

Sitosterol and stigmastanol are obtained from the Raney nickel catalyzed hydrogenation of stigmasterol. The sitosterol was separated from stigmastanol by silica gel column chromatography of its dibromide. Sitosterol was also obtained from the steam deodorizer distillate of crude cottonseed oil and purified by manifold recrystallizations of the free sterol from isopropanol and its acetate from alcohol-acetic acid mixtures at a low temperature.

INTRODUCTION

The isolation of pure sitosterol in quantity has always been a problem (1-4). Although it is the most widespread sterol in the plant kingdom, unlike the animal sterol cholesterol or the fungal sterol ergosterol, it usually occurs mixed with substantial quantities of similar compounds such as campesterol, stigmasterol and brassicasterol in mixtures that are readily separable on an analytical basis (5-7) but not on a preparative scale (8). The Sitosterol, N.F. available commercially is a 40:60 mixture of campesterol and sitosterol that is probably derived from crude soybean sterols after removal of stigmasterol (9).

Our work with insects requires pure sitosterol for itself and as a starting material for the preparation of 5,7-stigmastadien-3β-ol and 7-stigmasten-3β-ol (10). Sitosterol has been obtained by the partial hydrogenation of stigmasterol (4) or its 3,5-cyclo derivative (1), by chemical synthesis from pregnenolone (3), by degradation of impure material and resynthesis (2) and from natural sources by manifold crystallizations of the benzoate and acetate from ethyl acetate and alcohol (8). We report here the purification of sitosterol from two sources: (a) the mixture obtained by the selective hydrogenation of stigmasterol over Raney nickel (11); and (b) the steam distillate obtained from the deodorizer process in the commercial refining of cottonseed oil.

The steam deodorization process concentrates the sterols in vegetable oils. Crude oils derived from the olive, cottonseed, soybean, peanut, palm kernel, rapeseed, sunflower seed and linseed contain 0.1-0.45% sterols (12-14) and are in themselves poor sources of these compounds. During the deodorization process, however, the sterols are selectively removed from the mass of triglycerides in the oil by superheated steam in a vacuum so that their concentrations in these steam distillates are greatly augmented (15,16). A search of the literature (13,17) suggested that the steam distillates from olive and cottonseed oils would be the best sources of sitosterol. These oils have 98 and 96%, respectively, of their sterol fraction as sitosterol, the remainder being campesterol.

Deodorizer distillates of olive oil obtained from California and Greece proved to be poor sources of sitosterol. The sterol concentrations in these products were low and the squalene concentrations were very high, so that it was impractical to isolate sitosterol from them. We accordingly turned to the deodorizer distillate from the processing of cottonseed oil and found it to be an excellent source of sitosterol.

MATERIALS, METHODS AND RESULTS

Analysis

The analyses for campesterol, sitosterol, stigmasterol and stigmastanol were performed by gas liquid chromatography (GLC) and thin layer chromatography (TLC) as described in a following communication (18). In addition, sterols were separated from steryl esters by TLC on silica gel with cyclohexane-ethyl acetate 60:40.

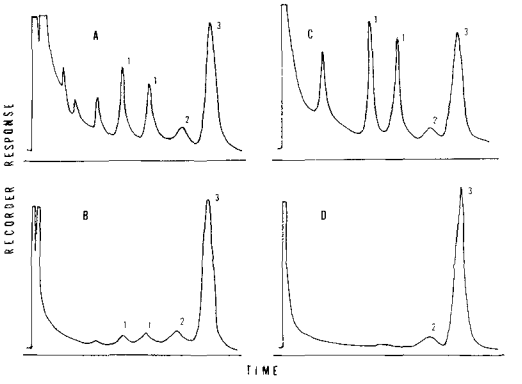

FIG. 1. Gas liquid chromatography diagrams: (1) tocopherols, (2) campesterol, (3) sitosterol. A. Total steam deodorizer distillate from cottonseed oil, B. solids from A, C. filtrate from A, D. crystals from B after crystallization from ethanol.

[1]Contribution No. 1946, Arizona Agricultural Experiment Station.

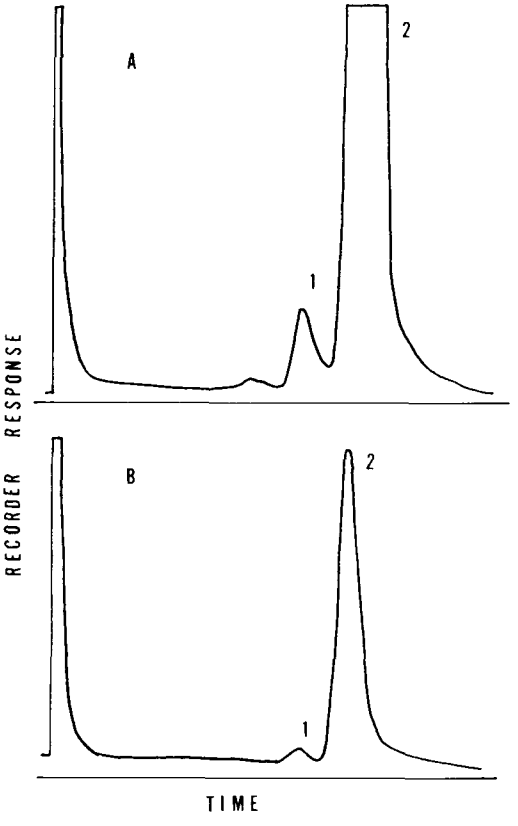

FIG. 2. Gas liquid chromatography diagrams: (1) campesterol, (2) sitosterol. A. Fraction Z, 5 μl (50 mg/ml), B. fraction Z, 1.4 μl (50 mg/ml).

Melting Points

Melting points were taken in vacuo; corrected.

Deodorizer Distillates

Deodorizer distillates from cottonseed were obtained from Ranchers Cotton Oil Co., Fresno, Calif., and those from olive from Wilsey Foods, Inc., Lindsay, Calif., and from Elais Oleaginous Products, S.A., Neon Faliron, Pireaeus, Greece.

Sitosterol from the Selective Hydrogenation of *Stigmasterol*

A typical hydrogenation of stigmasterol over Raney nickel (11) gave a product having the composition: 67% sitosterol, 31% stigmastanol, 2% stigmasterol (18). Twelve grams of this mixture in 200 ml ether was treated dropwise with 4.0 g Br_2 in 50 ml ether at room temperature and then placed in a freezer at -20 C for 3 days. After removal of 1.7 g stigmastanol, the filtrate was concentrated in vacuo without heat and the residue chromatographed on a 400 g silica gel column with ether-low boiling petroleum ether 1:4. The first four fractions (150 ml each) contained nothing, the next six contained 5.5 g sitosterol dibromide and the last six were composed of the dibromide and stigmastanol. The latter were rechromatographed in the same way to yield more of the dibromide free of the stanol. The combined dibromides were refluxed 4 hr in 250 ml ethanol-acetic acid 1:1 over 5 g Zn dust to yield 5.1 g sitosterol after recrystallization from acetone. This product, mp 136-7 C, was completely free of stigmastanol and showed only a trace of stigmasterol by GLC (Fig. 3A). The overall yield of purified sitosterol was 63% of that present in the original hydrogenation mixture.

Sitosterol from Cottonseed Oil Steam Deodorizer Distillate

Five gallons of the semisolid distillate (Fig. 1A) were filtered through three large, coarse porosity, sintered glass funnels with an oil pump vacuum. The filtration, which took several weeks, was hastened by daily scraping of the filter cake from the surface of the sintered glass with a large, flat-ended spatula. When the filter cake had been reduced to 6 liters, it was removed, melted on a steam bath and allowed to recrystallize. These crystals were filtered again for a week to remove another 3 liters of liquid. The resulting solid (2840 g, Fig. 1B) was saved for purification; the combined filtrates (Fig. 1C) were discarded. The solid was crystallized from 4 liters ethanol to remove remaining tocopherols to yield 2 kg of a mixtue of campesterol, sitosterol and steryl esters (Fig. 1D). In our system, the esters were not readily eluted from the GLC column. They were detected by a high R_f spot on TLC.

Five gram samples of the solid mixture were crystallized from 40 different solvents to determine which solvent would be the best for purification of sitosterol. The results were assayed by GLC and TLC as well as weight of solid recovered. Ten solvents (pyridine, dioxane, 2-butanone, ethyl acetate, tetrahydrofuran, *n*-amyl alcohol, 2-methyl-2-butanol, acetophenone and triethylamine) selectively preciptiated 0.1-0.5 g of the steryl esters from the 5 g mixture. These precipitates were combined (3 g) and recrystallized three times from *t*-butanol to yield a material, mp 85.5-87 C, that showed only one high R_f spot on TLC. It was hydrolyzed with alcoholic KOH to give only campesterol and sitosterol in the neutral fraction and palmitic acid in the acid fraction (GLC methyl ester, mp methyl ester 32-33 C, lit [19] 30 C).

A number of solvents (acetic acid, dimethyl-

formamide, chloroform, isopropyl alcohol, 2-ethoxyethanol and di-isobutyl ketone) appeared to precipitate sitosterol with some enrichment over its concentration in the original mixture. Of these, isopropyl alcohol was chosen for large scale work because of the yield of crystals (1.45 g at room temperature from 5 g in 100 ml solvent) and the ease of concentration and recovery when working with this alcohol.

The 2 kg of crude sterol were then saponified in and extensively crystallized and recrystallized from isopropyl alcohol. Twenty times as much solvent as sterol was routinely used. The sterol was dissolved on a steam bath in 20 liter cylindrical Pyrex water bath jars and allowed to crystallize at room temperature. Filtrates were concentrated and second and third crops obtained. The crystalline precipitates were assayed by GLC: 50 mg was dissolved in 1 ml benzene; 5 μl of this solution was injected, and the size of the campesterol peak measured in arbitrary units (Fig. 2A, B).

As the crystallizations, combination of similar fractions, and recrystallizations proceeded, the size of the campesterol peak gradually diminished until further crystallizations from isopropyl alcohol caused no decrease in its size relative to the large sitosterol peak. At this point the purified sitosterol fractions were acetylated. They were mixed with equal weights of acetic anhydride and of benzene and evaporated to dryness in large crystallizing dishes on a steam bath in the hood. The acetates were then crystallized and recrystallized from absolute ethanol, isopropanol and mixtures of the two alcohols with acetic acid.

It was more advantageous to recrystallize the sitosteryl acetates from dilute solution in the cold room at 4 C (ethanol-isopropanol-acetic acid, 1:1:1, 80-100 ml solvent/gram acetate) than from a more concentrated solution (20-50 ml/gram) at room temperature. The solubility vs. temperature curves of campesteryl and sitosteryl acetates are apparently not parallel lines.

When the process was arbitrarily halted after a total of 160 recrystallizations of the free sterols and steryl acetates, the six products shown in Table I were on hand. The purities of the sitosteryl acetates were estimated by GLC. The sizes of their campesteryl acetate peaks were compared to those of standards that were prepared by addition of 0.5, 1, 2, and 3% campesteryl acetate to samples of fraction 42.

A portion of fraction 42 was hydrolyzed to sitosterol, melting point 138-139 (Fig. 3B). Recent reported values for the melting point of sitosterol are 139-140 C (1,2); 136-137 C (4); 137-138 C (8).

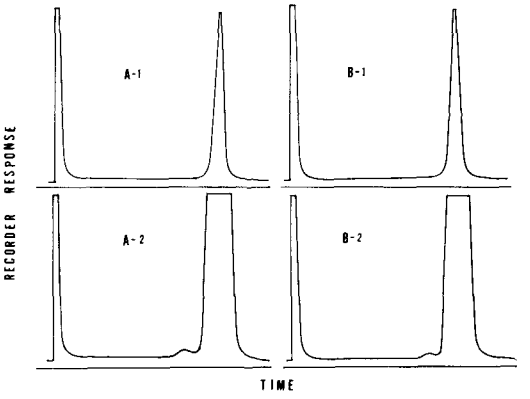

FIG. 3. Gas liquid chromatography diagrams. Purified sitosterol from: A. selective hydrogenation of stigmasterol (1. 1.4 μl [50 mg/ml]; 2. 5 μl [50 mg/ml]); B. cottonseed oil steam deodorizer distillate (1. 1.4 μl [50 mg/ml]; 2. 5 μl [50 mg/ml]).

DISCUSSION

Two simple techniques have been described for the isolation of sitosterol from inexpensive starting materials. The first uses the selective hydrogenation of stigmasterol to sitosterol over Raney nickel under mild conditions (11) with subsequent separation of sitosterol from stigmastanol by bromination and silica gel column chromatography. This method is appropriate for the preparation of gram quantities of sitosterol and is thereby more convenient than the preparative TLC plate separation of the mixture of sterol propionates obtained by the Pd-catalyzed hydrogenation of stigmasterol (4).

The second method, perhaps more useful in the preparation of centigram quantities of the sterol, takes advantage of the large amount of sitosterol present in the steam deodorizer distillates from cottonseed oil processing and the selectivity of isopropyl alcohol for the purification of sitosterol from contaminating campesterol. Although both methods are time consuming, they are easy to carry out with simple apparatus and they have provided us with the sitosterol that we need for insect feeding studies and further chemical modification.

The presence of steryl esters in the cottonseed oil deodorizer distillate was unexpected. Although the ratio of campesterol to sitosterol in the esters was the same as that found as free sterols, the absence of fatty acids other than palmitate was surprising. Possibly sterol esters of oleate and linoleate, two other acids found in cottonseed oil, were eliminated in the early filtration processes and only the sterol palmi-

TABLE I

Fractions of Sitosteryl Acetate Obtained from the Steam Distillate of Cottonseed Oil

Fraction no.	Weight, g	Melting point, C	Estimated purity
42	100	120.8-121.5	99.5%
61	99	121 -122	99.0%
64	141	121 -122	98.5%
65	102	121.5-122	98.0%
66	127	122 -123.2	97.5%
67	114	122.5-124	96.0%

tates were sufficiently high melting and insoluble to be carried over in our sterol fraction.

Another unexpected result was the increase in the melting point of sitosteryl acetate with an increase in its campesteryl acetate content (Table I). Similar results were obtained with the artificial mixtures of the two steryl acetates used to estimate the purities of the products in Table I. Addition of 0.5, 1, 2 and 3% campesteryl acetate to fraction 42 raised its melting point to 121-121.6, 121-121.7, 121-122 and 121-122.3 C, respectively. In these cases, the presence of an impurity raised rather than lowered the melting point of a compound. The reasons for this may be the close structural similarity between the two sterol acetates and the higher melting point (158-159 [8]) of the campesterol derivative. The two acetates appear to form an ideal solid solution in the concentration range that we investigated.

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REFERENCES

1. Steele, J.A., and E. Mosettig, J. Org. Chem. 28:571 (1963).
2. Fujimoto, G.I., and A.E. Jacobsen, Ibid. 29:3377 (1964).
3. Ikan, R., A. Markus and E.D. Bergmann, Ibid. 36:3944 (1971).
4. Allais, J.P., and M. Barbier, Qual. Plant. Mater. Veg. 16:215 (1968).
5. Patterson, G.W., Anal. Chem. 43:1165 (1971).
6. Tu, C., W.D. Powrie and O. Fennema, J. Food Sci. 35:601 (1970).
7. de Souza, N.J., and W.R. Nes, J. Lipid Res. 10:240 (1969).
8. Nishioka, I., N. Ikekawa, A. Yagi, T. Kawasaki and T. Tsukamoto, Chem. Pharm. Bull. (Tokyo) 13:379 (1965).
9. Poulos, A., J.W. Greiner and G.A. Fevig, Ind. Eng. Chem. 53:949 (1961).
10. Heed, W.B., and H.W. Kircher, Science 149:758 (1965).
11. Kircher, H.W., and F.U. Rosenstein, Lipids 8:101 (1973).
12. Karleskind, A., Rev. Fr. Corps Gras 14:251 (1967); 15:379 (1968).
13. Eisner, J., and D. Firestone, J. Ass. Off. Agr. Chem. 46:542 (1963).
14. Fedelli, E., A. Lanzani, P. Capella and G. Jacini, JAOCS 43:254 (1966).
15. Hoffman, R.L., C.D. Evans and J.C. Cowan, Ibid. 41:116 (1964).
16. Nelson, J.P., and A.J. Milun, Ibid. 45:848 (1968).
17. Karleskind, A., F. Audiau and J.P. Wolff, Rev. Fr. Corps Gras 13:165 (1966).
18. Rosenstein, F.U., and H.W. Kircher, Lipids 8:107 (1973).
19. "Handbook of Chemistry and Physics," 48th Edition, 1968.

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Hydrogenation of Stigmasterol[1]

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ABSTRACT

Hydrogenations of stigmasterol over 5% Pd/C, 5% Ru/C, Pt, Raney Ni, Raney Co, Ni/kieselguhr, and tris(triphenylphosphine)chlororhodium catalysts and with diimide generated from hydrazine and *p*-toluenesulfonylhydrazide were studied to determine the best conditons for the selective reduction of the Δ^{22} double bond. The best results (maximum stigmasterol reduction, minimum stigmastanol formation) were obtained with Raney nickel at room temperature and atmospheric pressure.

INTRODUCTION

The selective hydrogenation of the *trans*-Δ^{22} double bond of stigmasterol (5, 22-stigmastadien-3β-ol) yields sitosterol (5-stigmasten-3β-ol) (1-4). In two cases studied, the Δ^5 unsaturation was blocked by formation of the *i*-sterol before reduction (1,2), and in the other two, palladium-catalyzed hydrogenation was stopped after absorption of 1 mol H_2 (3,4). Sitosterol was separated in low yield from unreacted stigmasterol and stigmastanol by complicated methods (3) or by argentation thin layer chromatography (TLC) of the sterol propionates (4). Since neither these methods nor a more recent synthesis of sitosterol (5) appeared suitable to us for preparation of the large quantities of the pure sterol we required for insect work, we decided to reinvestigate the selective hydrogenation of stigmasterol to sitosterol to determine the optimal parameters for the reaction. In this communication we report our experiences with various catalysts and conditions and with two diimide generating reagents.

MATERIALS AND METHODS

Catalysts

Raney cobalt and Raney nickel (#27 and #28 active catalysts in water, respectively, W.R. Grace and Co.) were solvent exchanged before use, and W-2 Raney nickel was prepared from the alloy (6). Tris(triphenylphosphine) chlororhodium (Strem Chemicals, Inc.), nickel on kieselguhr (Girdler catalysts, Chemetron Corp.), 5% Pd on C (Engelhard), 5% Ru on C and PtO_2 (Matheson, Coleman and Bell) were used as obtained.

Sterols

Stigmasterol (Upjohn Pharmaceutical Co.) was crystallized once from ethyl acetate, mp 169-70 (lit. [7] 170), after which it showed no impurities by gas liquid chromatography (GLC) or TLC. Stigmasteryl acetate was simply prepared by evaporation of a mixture of the sterol, acetic anhydride and benzene on a steam bath in the hood overnight. The solid residue was washed with methanol in a blender and crystallized from ethyl acetate, mp 143-4 (lit. [7] 144). Stigmasteryl trimethylacetate, mp 175-7, phenylacetate, mp 128-30, and benzoate, mp 163-4.5 (lit. [7] 160), were prepared from the acyl chlorides in pyridine and their purities checked by TLC. The trimethylacetate and phenylacetate have not been reported before. Their identity was based on the starting materials used and their chromatographic homogeneity.

Reactions

Atmospheric pressure hydrogenations were

[1]Contribution No. 1889 University of Arizona Agricultural Experiment Station.

TABLE I

Hydrogenation of Stigmasterol and Stigmasterol Esters over Pd[a]

Sample	Reaction time, hr	Product composition, %		
		Stigmasterol	Sitosterol	Stigmastanol
Stigmasterol	0.2	10	33	57
Stigmasterol acetate	0.2	2	35	63
Stigmasterol trimethylacetate	0.25	2	45	53
Stigmasterol benzoate	23.5	13	42	45
Stigmasterol phenylacetate	8.5	6	66	28

[a]Stopped with absorption of 1 mol H_2/mol sterol (measured manometrically).

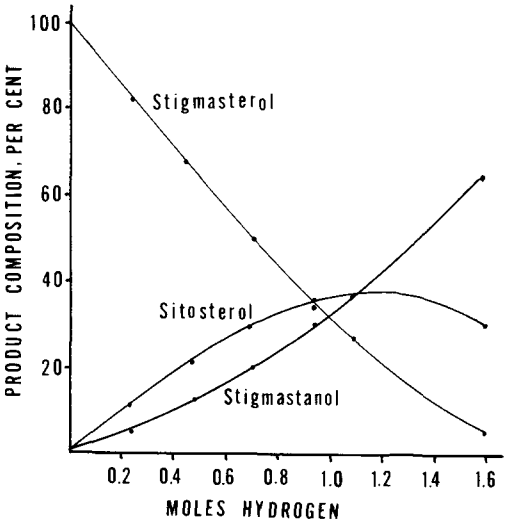

FIG. 1. Hydrogenation of 5 g stigmasterol over 0.5 g 5% Pd/C in 500 ml ethyl acetate.

run in small flasks on a magnetic stirrer connected to a mercury filled gas buret. Parr 1 and 2 liter stirred pressure vessels were used for the high temperature, high pressure runs. Reductions with hydrazine were done in conventional glassware and those with *p*-toluenesulfonyl hydrazide in 1 x 15 cm sealed Pyrex tubes in an oil bath. Additional details are given in the Results section.

Analysis

The analyses of the hydrogenation mixtures for stigmasterol, sitosterol and stigmastanol were done by GLC, TLC, and titration as described in the following communication (8). As a rule, catalysts were filtered from the sterol solutions which were then evaporated to dryness for analysis. Sterol esters were hydrolyzed with alcoholic KOH prior to analysis. The reactions with the soluble rhodium catalyst were evaporated and the sterols extracted from the catalyst with petroleum ether.

RESULTS

With Palladium

The reductions shown in Table I were run with 2 mmol sterol in 100 ml ethyl acetate over 0.4 g 5% Pd/C at room temperature and atmospheric pressure. When either 0.15 g $ZnCl_2$ or 2 drops 48% HBr were added as poisons to the mixture, hydrogenation did not occur. Reduction ran slowly at 2-3 C or when 0.2 drop 48% HBr was added, but in both cases, stigmastanol was still in the product. A quantitative assay for stigmasterol and its two reduction products is shown in Figure 1 as a function of hydrogen uptake. All points were from a single reaction mixture that was sampled over a period of 2 hr. The hydrogen uptakes were calculated from the sterol analyses rather than manometric measurements. The latter were misleading because of the rapidity of the reaction when run under the conditions described for Table I.

With Raney Nickel

The commercial Raney nickel after solvent exchange (water → ethanol → ethyl acetate) was ca. 25% as active as the catalyst (W-2) prepared in our laboratory from the alloy, as far as rate of hydrogenation was concerned. The product composition from the two catalysts was similar, however, and they were used interchangeably. Quantitative results from atmospheric pressure, room temperature runs are given in Table II and Figure 2.

Many experiments were performed to determine conditions of minimal stigmastanol and maximum sitosterol formation during the nickel catalyzed hydrogenation. Among these were additions of poisons such as $ZnCl_2$, pyridine and triphenylphosphine, which at high concentrations (50-500 mg/100 ml) completely inhibited reductions, and at low concentrations (4-30 mg/100 ml) did not prevent stigmastanol formation. Others included different solvents such as cyclohexane, dioxane, tetrahydrofuran (slow reactions, stigmastanol produced) and methylene chloride (no reaction). Inactivation of the Raney nickel by vacuum drying or using water wet catalyst without solvent exchange prevented hydrogenation. Very slow reductions were observed with a 2-year-old catalyst or when the reaction mixture was placed in an ice bath. In both cases stigmastanol was still detected in the reaction product.

With Raney Cobalt

No reduction of stigmasterol over this catalyst occurred under mild (4 days, room temperatrure, atmospheric pressure) or forcing conditions (18 hr, 100 C, 500 psig H_2). Raney cobalt was not a poison for the nickel-catalyzed reaction; when the latter was added to the mixture run under mild conditions, hydrogenation proceeded as usual.

With Supported Nickel on Kieselguhr

Stigmasterol (44 g) in 1500 ml absolute ethanol was reduced over 4 g Girdler G-49B catalyst (100 C, 500 psig H_2). Samples were removed periodically and analyzed by GLC and TLC. After 18 hr 90% of the stigmasterol had reacted. However stigmastanol was present in

TABLE II

Hydrogenation of Stigmasterol and Stigmasterol Esters over Raney Nickel[a]

Sample	mmol	Reaction time, hr	Product composition, %		
			Stigmasterol	Sitosterol	Stigmastanol
Stigmasterol[b]	2	9	2	64	34
Stigmasterol acetate	1	20	12	80	8
Stigmasterol trimethylacetate	1	18	66	31	3
Stigmasterol phenylacetate	1	29	0	80	20
Stigmasterol[c]	12	18	4	77	19
Stigmasterol	12	18	2	77	21
Stigmasterol	12	18	2	71	27
Stigmasterol	12	18	0	71	29

[a]Stopped with absorption of 1 mol H_2/mol sterol or after running overnight.
[b]Small scale reductions: 1 g (dry basis) #28 Raney nickel.
[c]Larger scale reductions: 3 g (dry basis) #28 Raney nickel.

all samples, even the one taken 1 hr after the start of the reaction.

The reaction was repeated with 4 g Girdler G-67RS catalyst (110 C, 1200 psig H_2). After 4 days 90% of the stigmasterol had been reduced, but again, every sample taken during the reaction showed stigmastanol to be present.

With Platinum

A platinum oxide catalyst whose activity had been reduced by two previous hydrogenations, cholesterol to cholestanol (9) and stigmasterol to stigmastanol, was refluxed in ethyl acetate, washed and suspended in fresh solvent. It was no longer active, even after addition of perchloric acid (9).

With Ruthenium

Stigmasterol (400 mg in 100 ml ethyl acetate) was stirred with 1 g 5% Ru/C under hydrogen for 18 hr. GLC showed that 40% of the starting material was still unreduced, and TLC indicated that a substantial amount of stigmastanol had formed.

With Tris(triphenylphosphine)chlororhodium

No reduction of stigmasterol in the presence of this catalyst took place under mild conditions. This was expected by analogy with ergosterol in which only the Δ^5 bond in a conjugated $\Delta^{5,7}$ system is reduced (10). The results from experiments run under forcing conditions are shown in Table III. In the first two experiments, where no reduction was observed, the IR spectrum of the recovered stigmasterol was superimposable on that of the starting material suggesting that no double bond migration or isomerization (*trans* → *cis*) had occurred (11).

With Hydrazine Hydrate

Several pilot experiments made by bubbling air through a hot solution of stigmasterol, hydrazine hydrate and acetic acid in propanol (12) showed that some sterol reduction took place. Stigmasterol (4.3 g) in 250 ml 2-methoxyethanol and 10 ml acetic acid was aerated vigorously for 9 days on the steam bath. During this time a total of 140 ml hydrazine hydrate was gradually added, together with sufficient solvent to keep the mixture homogenous. Evaporation to dryness followed by crystallization of the residue from alcohol yielded 1.7 g sitosterol that still contained ca. 10% of unreacted starting material.

With *p*-Toluenesulfonyl Hydrazide

Solutions of stigmasterol (0.1 mmole), *p*-tosylhydrazide (1 mmole) and either ethanol-

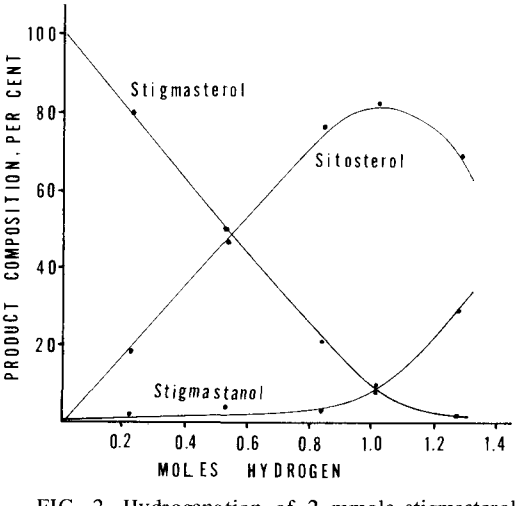

FIG. 2. Hydrogenation of 2 mmole stigmasterol over 1.2 g (dry basis) #28 Raney nickel in 100 ml ethyl acetate. (Each set of three points was a separate run.)

TABLE III

Hydrogenation of Stigmasterol over Tris(triphenylphosphine)chlororhodium

Experiment	Sterol wt, g	Catalyst wt, g	Reaction medium[a]	Temperature, C	Psig H_2	Time, days	Results
1	1.25	0.25[b]	100 ml benzene	30	900	2.5	No reduction
2	1.0	0.25[b]	100 ml benzene-ethanol 3:1	80	1000	1	No reduction
3	44	3.0[b]	1000 ml benzene-ethanol, 3:1, 25 ml Et_3N	125	1000	6[c]	30% unreacted stigmasterol
				60	1400	1	No reduction
				150			
4	38	3.0[d]	1000 ml benzene, 10 ml Et_3N	150	1400	1	No reduction
			350 ml ethanol, 25 ml Et_3N added	150	1400	8[d]	78% unreacted stigmasterol

[a]Solvents purged with H_2 before use; benzene purified to remove thiophene.
[b]Catalyst prereduced with H_2 for 20 min before sterol addition.
[c]Catalyst decomposed.
[d]Catalyst prereduced with H_2 2 hr/1200 psig before sterol addition.

amine (1 mmole) or triethylamine (1 mmole) in ethyl 2 ml of 2-methoxyethanol, 1,2-dimethoxy ethane or diethyleneglycol dimethyl ether were heated at 80 or 105 C for 17 and 44 hr in sealed tubes (13,14). The tubes were cooled, opened, water added and the sterols extracted with benzene-petroleum ether 1:1 for analysis by GLC and TLC. Only traces of stigmastanol were observed, but reduction of stigmasterol occurred only from 17 to 31%. Best results were obtained with triethylamine catalyst in 1,2-dimethoxyethane and 2-methoxyethanol. Only small differences were seen between the experiments run at 80 and 105 C and between the 17 and 44 hr runs.

The results from an experiment to determine the effect of increasing the amount of reducing agent are shown in Table IV. The reactions were run in 2-methoxyethanol with a 1:1 mole ethyl ratio of triethylamine to *p*-tosylhydrazide for 20 hr at 80 and 105 C. All reaction mixtures showed traces of stigmastanol by TLC.

DISCUSSION

All of the stigmasterol hydrogenations over palladium, whether poisoned or not, at room or ice bath temperatures, yielded substantial amounts of stigmastanol even when much unreacted stigmasterol was still present. The steric effect of 3β-acyloxy groups is evident during the bromination of cholesteryl esters. Cholesterol trimethylacetate, acetate and benzoate react only 18, 15 and 11% as rapidly with bromine as the free sterol (15). When these derivatives of stigmasterol were hydrogenated over palladium, however, the acyl groups did not appreciably hinder reduction of the Δ^5 bond. Palladium, used frequently in the past since the description by Bernstein and Wallis in 1938 (3), should no longer be considered effective for the selective hydrogenation of stigmasterol to sitosterol.

Less active catalysts such as 5% ruthenium on carbon and nickel on kieselguhr were not useful; stigmasterol was only slowly and unselectively reduced in their presence. Raney cobalt and a twice-used platinum catalyst were completely ineffective in promoting hydrogenation.

The hydrogenation of carbon-carbon double bonds in the presence of the soluble catalyst tris(triphenylphosphine)chlororhodium has been recently reviewed (11,16). Although it has been claimed that di- and not tri-substituted double bonds are reduced under mild conditions (17), the latter are reduced in the 1-methylcyclohexene series, albeit at a much slower, solvent dependent rate (18). Except for

TABLE IV

Reduction of Stigmasterol by *p*-Toluenesulfonyl Hydrazide

mmol Hydrazide and amine per 0.1 mmol sterol	Amount unreacted stigmasterol as estimated by gas liquid chromatography, % of product	
	at 80 C	at 105 C
1	68	70
2	66	68
3	60	65
4	57	66
5	55	65

the facile hydrogenation of Δ^1, Δ^2, Δ^3-cholestenes (19), α,β-unsaturated ketones (10,19) and the reduction of ergosterol to the 5,6-dihydroderivative (10), the hydrogenation of other double bonds in the sterol molecule has not been described. We also have been unable to reduce either the Δ^5 or Δ^{22} double bond of stigmasterol under mild conditions. In the presence of mixed solvents, forcing conditions, and a promoter such as triethylamine (20), some reduction of stigmasterol to sitosterol was observed. Little, if any, stigmastanol was produced in these reactions, and possibly with different ligands than triphenylphosphine, such as the recently described diphenylpiperidinophosphine (18), tri-*t*-butylphosphine (21), or the bis(pyridine)-dimethylformamidoborohydride complex (22), a satisfactory selective hydrogenation of stigmasterol can be achieved with a soluble rhodium catalyst.

Hydrogenations with diimide, whether generated from hydrazine or *p*-tosyl hydrazide have been described as selective (14,23), and the reduction of stigmasteryl acetate is mentioned in a review (24) as unpublished work with no details given. In our experiments, stigmasterol hydrogenation was too incomplete, even with large excesses of either reagent, to warrant the use of diimide as a reductant. Also, even though much stigmasterol still remained in the reaction mixtures, small amounts of stigmastanol were already apparent in them.

Our best results were obtained with Raney nickel. This catalyst allowed almost complete reduction of the Δ^{22} bond of stigmasterol before appreciable Δ^5 reduction occurred (Fig. 2). It was far better than palladium in selectivity (compare Fig. 1) and can be used with fairly consistent results (Table II, lower part). The selectivity may be improved by derivative formation (Table II, upper part); the diphenylacetate is a good candidate for future studies. The isolation of pure sitosterol from a Raney nickel-catalyzed reduction of stigmasterol is the subject of the preceding report (25).

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REFERENCES

1. Fernholz, E., and W.L. Ruigh, J. Amer. Chem. Soc. 62:3346 (1940).
2. Steele, J.A., and E. Mosettig, J. Org. Chem. 28:571 (1963).
3. Bernstein, S., and E.S. Wallis, Ibid. 2:341 (1937).
4. Allais, J.P., and M. Barbier, Qual. Plant. Mater. Veg. 16:215 (1968).
5. Ikan, R., A. Markus and E.D. Bergmann, J. Org. Chem. 36:3944 (1971).
6. Org. Syn., Coll. Vol. III, p. 181.
7. Shoppee, C.W., "Chemistry of the Steroids," Second edition, Butterworths, London, 1964, p. 45, 63.
8. Rosenstein, F.U., and H.W. Kircher, Lipids 8:107 (1973).
9. Hershberg, E.B., E. Oliveto, M. Rubin, H. Staeudle and L. Kuhlen, J. Amer. Chem. Soc. 73:1144 (1951).
10. Birch, A.J., and K.A.M. Walker, J. Chem. Soc. "C" 1966:1894.
11. Augustine, R.L., and J.F. Van Peppen, JAOCS 47:478 (1970); J. Chem. Soc. "D" 1970; 495.
12. Asinger, F., B. Fell, G. Hadik and G. Steffen, Chem. Ber. 97:1568 (1964).
13. Garbisch, E.W., Jr., S.M. Schildkraut, D.B. Patterson and C.M. Specher, J. Amer. Chem. Soc. 87:2932 (1965).
14. Hunig, S., H.R. Muller and W. Thier, Angew. Chem., Int. Eng. Ed. 4:271 (1965).
15. Schwartz, V., S. Hermanek and J. Trojanek, Coll. Czech. Chem. Comm. 26:1438 (1961).
16. Lyons, J.E., L.E. Rennick and J.L. Burmeister, Ind. Eng. Chem. Prod. Res. Develop. 9:2 (1970).
17. Biellemann, J.F., and H. Liesenfelt, Compt. Rend. "C" 263:251 (1966).
18. Hussey, A.S., and Y. Takeuchi, J. Amer. Chem. Soc. 91:672 (1969); J. Org. Chem. 35:643 (1970).
19. Djerassi, C., and J. Gutzwiller, J. Amer. Chem. Soc. 88:4537 (1966).
20. Morrison, J.D., R.E. Burnett, A.M. Aguiar, C.J. Morrow, C. Phillips, Ibid. 93:1301 (1971).
21. Masters, C., W.S. McDonald, G. Raper and B.L. Shaw, J. Chem. Soc. "D" 1971; 210.
22. Abley, P., F. Jardine and F.J. McQuillin, J. Chem. Soc. "C" 1971:840.

23. Ohno, M., and M. Okamoto, Tet. Lett. 35:2423 (1964); H. Nozaki and R. Noyori, J. Org. Chem. 30:1652 (1965).
24. Aylward, F., and M. Sawitowska, Chem. Ind. 1962:484.
25. Kircher, H.W., and F.U. Rosenstein, Lipids 8:97 (1973).

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Analysis of Mixtures of Stigmasterol, Stigmastanol and Sitosterol[1]

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ABSTRACT

Hydrogenation of stigmasterol yields mixtures of the three title sterols. Stigmasterol was clearly separated from the other two by gas liquid chromatography; a standard curve was constructed relating the height of its peak to its concentration in the mixture. The total unsaturation was measured by a dibromopyridine sulfate-thiosulfate procedure. The unsaturation contributed by previously determined stigmasterol was subtracted from the total to leave that contributed by sitosterol, whose concentration in the mixture was then calculated. The amount of stigmastanol, whose presence was qualitatively determined by thin layer chromatography after bromination of the mixture on the plate, was obtained by difference. When suitable corrections were made, based on analysis of known mixtures, the concentrations of three sterols in hydrogenation mixtures could be calculated.

INTRODUCTION

We required a method for the determination of the content of stigmasterol, sitosterol and stigmastanol in mixtures that were obtained by the catalytic hydrogenation of stigmasterol (1). The separation of stigmasterol from a number of other sterols by gas liquid chromatography (GLC) has been amply described (2, and references cited therein), and quantitative GLC methods have been published (3,4). The separation of stigmastanol from sitosterol is less well documented, and although a separation factor of 1.13 has been claimed for these two sterols on neopentylglycol succinate (5), the separation of sitosterol from stigmasterol on this phase was poor (separation factor 0.97). We found no liquid phase that would separate the three title sterols sufficiently from each other that would allow us to use it as a basis for a quantitative GLC determination of the three. We therefore turned to a combination of GLC, thin layer chromatography (TLC) and titrimetric procedures to achieve our goal.

MATERIALS AND METHODS

Sterols

The purification of stigmasterol and its esters has been described (1). Stigmastanol was prepared by hydrogenation of stigmasterol over PtO_2 (6) and crystallized from acetone (negative Lieberman-Burchard reaction). Sitosterol was isolated from a mixture obtained by partial hydrogenation of stigmasterol over Raney nickel (7). Cholesterol, USP (Nutritional Bio-

[1]Contribution No. 1902, Arizona Agricultural Experiment Station.

TABLE I

Titration of Sterol Double Bonds with Dibromopyridine Sulfate and Sodium Thiosulfate

Sterol	Melting point range	Double bonds per molecule
Cholesterol	147.5 - 148.5	1.004
Cholesterol acetate	113.5 - 114.5	0.992
Cholesterol propionate	94 - 97	0.994
Cholesterol isobutyrate	127 - 129	1.002
Cholesterol pivalate	160.5 - 161.5	0.993
Cholesterol benzoate	148.5 - 150	0.984
Sitosterol	136 - 137	0.998
Stigmastanol	135 - 137	-0.004[a]
Stigmasterol	169 - 170	2.15[b]
Stigmasterol acetate	142.5 - 144	2.122
Stigmasterol pivalate	175 - 177	2.195
Stigmasterol benzoate	163 - 164.5	2.158
Stigmasterol phenylacetate	128 - 130	2.165

[a]Sample consumed more thiosulfate than blank.

[b]Average of six values; see text.

TABLE II

Analyses of Known Mixtures

	Sterol mixture	Concentration as prepared, wt%	Concentration as found, wt%		Error, %
			From gas liquid chromatography[a]	From titration	
A	Stigmasterol	35.3	35.0		-0.3
	Sitosterol	33.9	65.0	33.5	-0.4
	Stigmastanol	30.8		31.5	+0.7
B	Stigmasterol	5.3	5.0		-0.3
	Sitosterol	68.0	95.0	66.2	-1.8
	Stigmastanol	26.7		28.8	+2.1
C	Stigmasterol	33.5	33.0		-0.5
	Sitosterol	27.7	67.0	29.9	+2.2
	Stigmastanol	38.8		37.1	-1.7
D	Stigmasterol	66.5	66.5	67.2	+0.7
	Stigmastanol	33.5	33.5		

[a]Silylethers on QF-1; stigmasterol concentrations in mixtures A, B and C determined with line c in Fig. 1; and in mixture D, with line b in Fig. 1.

chemicals Co.), was recrystallized twice from ethyl acetate and its esters prepared in the usual way (1). All sterols and esters were dried in vacuo at 55 C prior to analysis. The pure compounds were chromatographically homogeneous; their melting point ranges are listed in Table I.

Gas Liquid Chromatography

Stainless steel columns (1/4 in. diameter, 6 ft long, silanized with dichlorodimethylsilane in benzene and washed with methanol) were packed with 5% OV-101 (4) or 3% QF-1 on Anakrom ABS for analysis of free sterols or silated derivatives, respectively. The former were run at 260 C/60 psig Ar and the latter at 235 C/36 psig Ar in the Research Specialties Co. Model 600 instrument, Sr^{90} detector. Both columns clearly separated stigmasterol from a mixture of sitosterol and stigmastanol. The peaks for the latter two sterols were superimposable on the OV-101 column; on the QF-1 column, stigmastanol had a slightly longer retention time.

Standard curves were constructed with known mixtures of stigmasterol and sitosterol, with stigmasterol and stigmastanol, and with stigmasterol and a 1:1 mixture of the other two sterols. We had problems relating the peak areas to concentrations, but when the height of the stigmasterol peak, as a ratio of its height to the height of both peaks, was plotted as a function of the stigmasterol content of the mixtures, straight lines were obtained (Fig. 1). Early work was performed on the QF-1 column with sterols that had been silated with N,O-bis(trimethylsilyl)acetamide; later when OV-101 was available, it was used with the free sterols. Routinely, a sample was dissolved in benzene and an appropriate amount injected. The heights of the stigmasterol peak (a) and the sitosterol-stigmastanol peak (b) were measured and the value a/a+b calculated. From this value the stigmasterol content of the mixture was obtained from Figure 1.

Thin Layer Chromatography

Stigmastanol was readily observed in the presence of the other two sterols by TLC on silica gel with benzene-ethyl acetate 4:1 (8) and overspotting of the sample with 10% Br_2 in chloroform on the plate before development (9). After development, the plate was heated at 110 C to reveal two green spots at higher R_f caused by the 5α, 6β and 5β, 6α dibromides of sitosterol and stigmasterol (10). These two sterols are not separated by this method, nor is it applicable to the separation of sterol and stanol acetates. Stigmastanol was subsequently revealed as a lower R_f spot (brown) when the plate was sprayed with 30% H_2SO_4 and heated at 110 C.

Determination of Unsaturation

Methods that have been described in the Russian literature (11,12) were adapted. The sterol samples (~0.15 g) in 20 ml chloroform in an ice bath were treated with a 0.1 N solution of dibromopyridine sulfate (13). Fifteen or 20 ml of reagent were used depending on sample size and degree of unsaturation, and blanks were run concurrently. The resulting solution was stirred at 2-3 C for 5 min after which time 20 ml 10% KI was added and stirring continued an additional 3 min in the cold. After addition of 100 ml water, excess bromine (as I_2) was determined by titration with 0.1 N sodium

thiosulfate and starch.

Calculations

The stigmasterol content of a sample was determined by GLC with the standard curve, and the total number of double bonds per mole sterol was determined by the titrimetric procedure. The unsaturation contributed by the stigmasterol was subtracted from the total to leave that contributed by sitosterol. Subtraction of the stigmasterol and sitosterol content from 100% gave the stigmastanol by difference. In the calculation, the number of double bonds contributed by stigmasterol was assumed to be the experimentally determined 2.15 double bonds per mole (Table I) rather than the theoretical value of 2.00. A typical calculation follows; the sample was obtained during the Pd-catalyzed hydrogenation of stigmasterol (1). Molecular weight of all three sterols was 415±2, average mol wt 415. Per cent sitosterol = ([ml] [N]/2) (100/sample wt, g/0.415) - (2.15) (Per cent stigmasterol) = (20.8) (ml) (N)/sample wt, g - (2.15) (per cent stigmasterol). Example: GLC determined stigmasterol = 36.0%; 0.1315 g sample equivalent to 6.94 ml 0.1014 N thiosulfate; per cent sitosterol = (20.8)(6.94)(0.1014)/0.1315-(2.15)(36.0) = 33.9%; per cent stigmastanol = 100 - 36.0 - 33.9 = 30.1%.

RESULTS AND DISCUSSION

The analysis of a number of sterols by the titrimetric procedure is shown in Table I. The double bonds per molecule of cholesterol and its derivatives, sitosterol and stigmastanol are all as expected within the margin of error of the method. When the bromination was run at room temperature instead of in an ice bath, the values were 4-6% too high. Titration of sterol double bonds with Br_2 in acetic acid at room temperature (12) also gave values that were too high, whereas the Br_2-Br^- in methanol reagent (14) gave low, inconsistent results.

Titration of stigmasterol under our conditions gave the consistently high value of 2.15 double bonds per sterol molecule (2.146, 2.122, 2.170, 2.156, 2.152, 2.164). These results were also reflected in the values obtained for stigmasterol derivatives (Table I). We have no explanation for this. There was no evidence that a triene was present in the stigmasterol. It would have to represent 15% of the stigmasterol to explain the anomalously high value and would be readily detectable by chromatographic methods.

GLC analysis of the silated derivatives on QF-1 required small corrections to be made

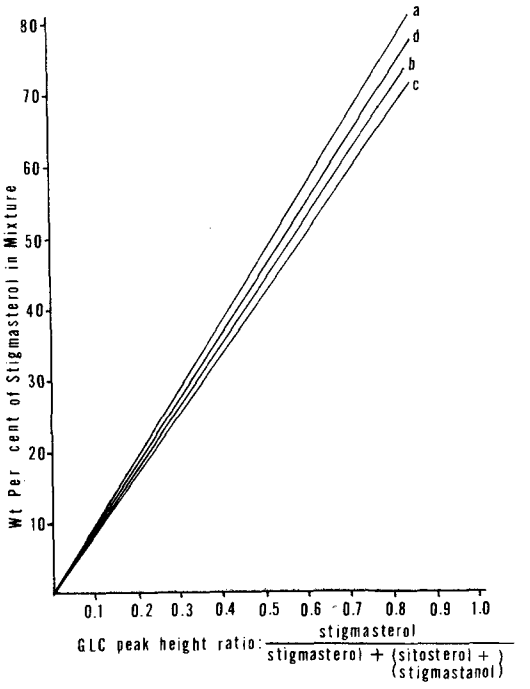

FIG. 1. Standard curves. a, b, c: QF-1 column, sterol trimethylsilyl ethers; d: OV-101 column, free sterols. (a) Stigmasterol-sitosterol; (b) stigmasterol-stigmastanol; (c), (d) stigmasterol-(sitosterol-stigmastanol) 1:1.

when stigmasterol concentration was to be determined. Because stigmastanol had a slightly longer retention time than sitosterol on this liquid phase, and perhaps because there was a difference in detector response between the two, the peak height ratio for the same stigmasterol concentration differed when sitosterol, stigmastanol or a mixture of the two constituted the remainder of the sample (Fig. 1). The peak contributed by the mixture was broader and lower than the equivalent amount of either compound taken singly. With the free sterols on the OV-101 column, sitosterol and stigmastanol had identical retention times and a sharp peak was observed for all mixtures of the two.

Qualitative determinations of saturated sterols by TLC have been made on silver nitrate-impregnated plates (15,16), after bromination of unsaturated sterols (8,9), and after oxidation of unsaturated sterols with *m*-chloroperbenzoic acid (17). A quantitative procedure for the determination of cholestanol by GLC after oxidation of unsaturated sterols (mainly cholesterol) with H_2O_2 in formic acid (18) was not useful for stigmastanol; our sterol mixtures were insufficiently soluble in the reagent. We found TLC after bromination on the plate to be the best way for detecting even very small

quantities of stigmastanol.

Results obtained by analysis of known mixtures of the three title sterols are shown in Table II. They show the method to be accurate to ±2% of the true content of each sterol in a mixture. The results obtained by analysis of hydrogenation mixtures are given in a previous report (1). They were useful to us for the determination of optimum conditions for the preparation of sitosterol from stigmasterol.

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REFERENCES

1. Kircher, H.W., and F.U. Rosenstein, Lipids 8:101 (1973).
2. Patterson, G.W., Anal. Chem. 43:1165 (1971).
3. Rosanski, A., Ibid. 38:36 (1966).
4. Grunwald, C., Anal. Biochem. 34:16 (1970).
5. Ikekawa, N., R. Watanuki, K. Tsuda and K. Sakai, Anal. Chem. 40:1139 (1968).
6. Hershberg, E.B., E. Oliveto, M. Rubin, H. Staeudle and L. Kuhlen, J. Amer. Chem. Soc. 73:1144 (1951).
7. Kircher, H.W., and F.U. Rosenstein, Lipids 8:97 (1973).
8. Ikan, R., S. Harel, J. Kashman and E.D. Bergmann, J. Chromatogr. 14:504 (1964).
9. Cargill, D.I., The Analyst 87:865 (1962).
10. Barton, D.H.R., and A.J. Head, J. Chem. Soc. 1956:932.
11. Khatletskii, A.M., and I.M. Yurist, Zhur. Obsch. Khim. 24:535 (1954); Chem. Abstr. 49:6288 (1955).
12. Perelman, Y.M., and G.F. Gavrilin, Zhur. Analit. Khim. 18:529 (1963).
13. Rowe, R.G., C.C. Furnas and H. Bliss, Ind. Eng. Chem., Anal. Ed. 16:371 (1944).
14. Byrne, R.E., Jr., and J.B. Johnson, Anal. Chem. 28:126 (1956).
15. Allais, J.P., and M. Barbier, Qual. Plant. Mater. Veg. 16:215 (1968).
16. Kammereck, R., W.H. Lee, A. Paliokas and G.J. Schroepfer, Jr., J. Lipid Res. 8:282 (1967).
17. Azarnoff, D.L., and D.R. Tucker, Biochim. Biophys. Acta 70:589 (1963).
18. Rosenfeld, R.S., Anal. Biochem. 12:483 (1965).

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Activation of Long Chain Fatty Acids by Subcellular Fractions of Rat Liver: I. Activation of *trans*-Unsaturated Acids

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ABSTRACT

The optimal fatty acid to protein ratio for maximum rat liver microsomal or mitochondrial activation of the *trans*-monounsaturated acids elaidate or *trans*-vaccenate is similar to the ratio for maximum palmitate activation but approximately double the optimal ratio for maximum activation of the *cis*-unsaturated fatty acids, oleate, *cis*-vaccenate, or of the *trans*-diunsaturated linelaidic acid. However, when the substrate is a fatty acid-albumin complex rather than free fatty acids, optimal fatty acid activation appears to be independent of geometrical configuration or degree of unsaturation. All-*trans* retinoate, d-α-tocopheryl acid succinate, and dihomogeranoate are not activated themselves, but at low concentrations extensively inhibit activation of *cis*-acids (oleate, *cis*-vaccenate, petroselenate, linoleate, linolenate, arachidonate) and the *trans*-diunsaturated linelaidate but do not affect activation of palmitate or *trans*-monounsaturated acids elaidate and *trans*-vaccenate. Anionic, cationic or nonionic detergents in a pH 7.4 buffered incubation medium were shown to inhibit, have no effect on, or apparently activate oleate activation, respectively, while not affecting or inhibiting palmitate and elaidate activation. The effect observed was dependent on the fatty acid to protein ratio, the fatty acid configuration and the detergent concentration.

INTRODUCTION

Most of the naturally occurring unsaturated fatty acids have the *cis*-configuration. However, when vegetable oils are hydrogenated, the *cis*-acids may be isomerized to *trans*-acids. A survey of various margarines showed that the fatty acids consisted of 18-55% *trans*-isomers (1). Previous studies of *trans*-acid metabolism have been concerned with acyl-transfer, cholesterol ester synthesis and hydrolysis, β-oxidation, absorption, incorporation into lipids and hydrolysis of lipids containing *trans*-acids (2-24). The present study was undertaken to delineate the parameters required for optimal *trans*-acid activation and to compare activation of *trans*-isomers with activation of saturated and *cis*-unsaturated long chain fatty acids. A brief abstract of this study has been published (25).

EXPERIMENTAL PROCEDURES

Fatty acids were purchased from the Hormel Institute, Austin, Minn. All-*trans* retinoic acid and d-α-tocopheryl acid succinate were purchased from Distillation Products Industries, Rochester, N.Y. Methyl dihomogeranoate was from W. Bowers, USDA, Beltsville, Md. Fatty acid free bovine albumin fraction V was the product of Pentex Biochemicals, Kankakee, Ill. Triton X-100 (polyoxyethylene alochol lauryl ether), sodium deoxycholate, sodium lauryl sulfate, ATP and coenzyme A were from Sigma Chemical Co. Cetyl pyridinium chloride was from Matheson, Coleman and Bell.

Preparation of Cell Fractions

Rat livers from male or female rats (Wistar strain, Microbiological Associates, Bethesda, Md.), 6-10 weeks of age and fed ad libitum on Purina rat pellets, were homogenized in 9 volumes of either 0.25 M sucrose or 0.25 M sucrose buffered to pH 7.4 with 50 mM Tris, 25 mM KCl and 5 mM $MgCl_2$. Mitochondria and microsomes were separated by differential centrifugation (26), and these cell fractions were stored frozen at -20 C until used.

Assay Procedure

Acyl-CoA formation was followed by hydroxamate-trapping (27). The standard assay system contained the following μmoles in a final volume of 1.0 ml; a mixture of NH_2OH, 500; Tris, 100; NaF, 25 neutralized to pH 7.4; $MgCl_2$, 4; cysteine-HCl, 30 neutralized with Tris, 40; ATP, 10; coenzyme A, 0.5; 400-850 μg microsomal or mitochondrial protein and 2-4 μmol fatty acid potassium salt. Incubation was for 30 min at 37 C. Control tubes lacking ATP and coenzyme A were always assayed, and the net hydroxamate formation was obtained by subtracting the control values from the values obtained with the incubation tubes containing the complete system. Activation of palmitate and *trans*-unsaturated acids was proportional to protein. For activation of *cis*-unsaturated acids, the ratio of protein to fatty acid was used that resulted in maximum activation

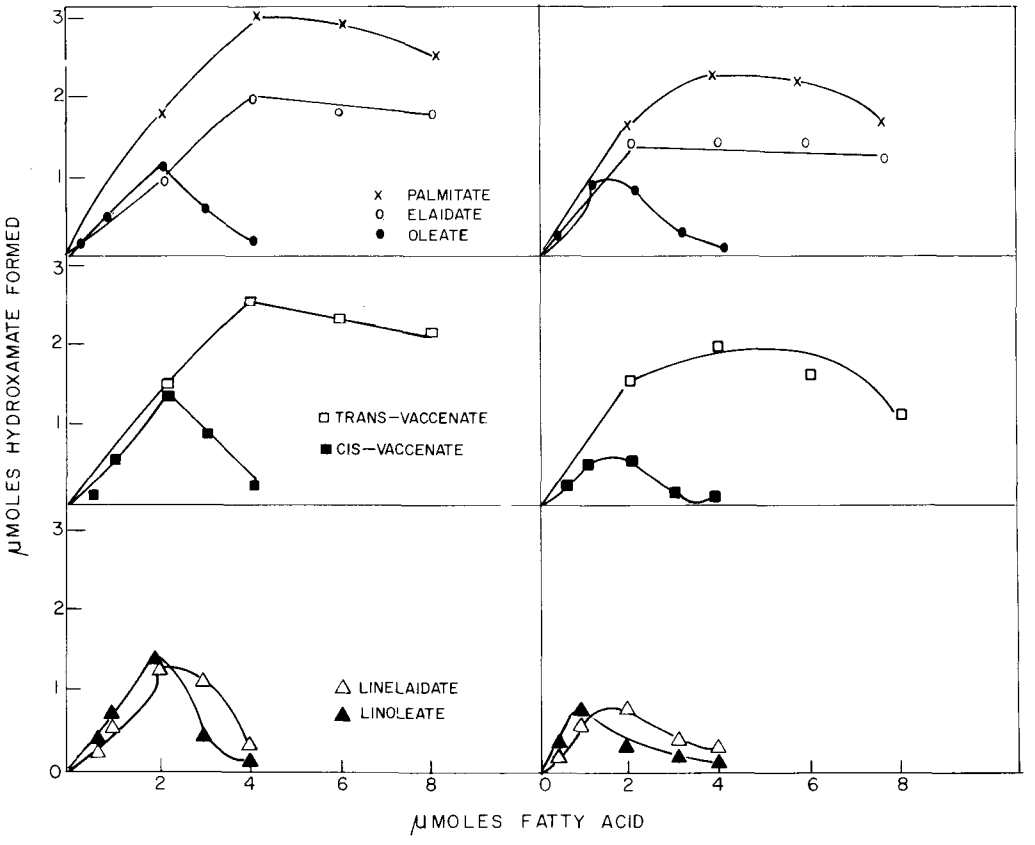

FIG. 1. Effect of fatty acid concentration on microsomal and mitochondrial activation of *cis*- and *trans*-unsaturated acids and palmitate. Each tube contained the components of the standard incubation mixture plus the concentrations of fatty acid indicated (X, palmitate; ○, elaidate; ●, oleate; □, *trans*-vaccenate; ■, *cis*-vaccenate; △, linelaidate; ▲, linoleate) and either 475 μg microsomal protein or 570 μg mitochondrial protein in a final volume of 1.0 ml. Incubation was for 30 min at 37 C. Results expressed as μmoles hydroxamate formed are shown for microsomes (left side) and mitochondria (right side).

(see text).

Protein was determined by the phenol method (28).

RESULTS

Optimal Requirements for Activation

Pande and Mead have previously shown that maximum activation of the *cis*-unsaturated acids oleate and linoleate occurs at a fixed fatty acid-protein ratio, and a decreased rate of activation is observed at ratios higher than the optimal ratio (27). In the present study, the optimal fatty acid-protein ratios for maximum microsomal and mitochondrial activation of *cis*-vaccenate, petroselenate (not shown), as well as that for the diunsaturated acids linoleate and linelaidate (microsomal), were in the same range as that reported previously for oleate (27). In contrast, the *trans*-isomers elaidate, *trans*-vaccenate and linelaidate (mitochondrial) exhibited higher optimal fatty acid-protein ratios for maximum activation than the ratios required for optimal activation of the corresponding *cis*-unsaturated acids. The *cis*-acids, as well as linelaidate, almost completely inhibited their own activation at the fatty acid-protein ratios required for maximum activation of the *trans* monounsaturated acids or plamitate. These results are shown in Figure 1 and summarized in Table I. The inhibitions caused by excess *cis*-acids were reversible and could be overcome by including more microsomal or mitochondrial protein in the incubation mixture. As shown in Table II, when the fatty acids were incubated as fatty acid-albumin complexes rather than as free fatty acids, maximum activation was observed in the presence of 2.6-4.9 mg albumin, independent of the *cis*- or *trans*-configuration or degree of unsaturation.

Effect of Positional Isomerism

Movement of the double bond position from a distance of 6 carbon atoms from the carboxyl group to 11 carbons from the carboxyl group did not markedly affect the rate of *cis*-acid acyl-coenzyme A formation. For a typical experiment using the standard incubation conditions, the microsomal activities found with petroselenate, oleate and *cis*-vaccenate (the *cis* Δ^6, Δ^9 and Δ^{11} octadecenoates) as substrates and expressed as μmoles of hydroxamate per hour per milligram protein were: 3.35, 3.37 and 3.56, respectively.

The rate of activation of the Δ^9 *trans* isomer (elaidate) was slightly lower than the rate of activation of the Δ^{11} *trans*-acid (*trans*-vaccenate). In two separate determinations, the average μmoles of hydroxamate formed per 30 min by the microsomal enzyme using the standard incubation conditions were for the Δ^9 and Δ^{11} isomers, respectively: 1.36 (± 0.05) and 2.30 (± 0.30). Analogous results were obtained with the mitochondrial enzyme.

Inhibitor Studies

All-*trans* retinoate was not activated by rat liver subcellular fractions. Sixty to eighty per cent inhibition of *cis*-vaccenate, oleate, linoleate and linelaidate was observed when 1 mM retinoate was included in an incubation mixture containing 636 μg microsomal protein and 2 μmol fatty acid. In contrast, activation of the *trans*-monounsaturated acids elaidate or *trans*-vaccenate, as well as activation of palmitate, was not inhibited under these conditions. These results are illustrated in Figure 2. Also, d-α-tocopheryl acid succinate strongly inhibited activation of *cis*-vaccenate and linoleate, and to a lesser extent inhibited the activation of linolenate and arachidonate, but did not inhibit the activation of palmitate, elaidate or *trans*-vaccenate. Dihomogeranoate inhibited oleate activation but did not affect the activation of palmitate (Table III). Similar selective inhibition of *cis*-unsaturated acid activation was observed when the mitochondrial fraction was used as the enzyme source in place of the microsomal fraction. The inhibition of oleate activation by retinoate could be overcome by addition of mitochondrial protein to the incubation mixture and resulted in S-shaped curves when activation was plotted against milligram protein (Fig. 3). Similar S-shaped activation curves were obtained for *cis*-vaccentate, linoleate and linelaidate with increasing protein. Retinoate also inhibited oleate activation when the substrate was an oleate-albumin complex rather than potassium oleate, but much higher concentrations of retinoate were then required to obtain the same degree of inhibition of oleate activation. For example, 8 μmol retinoate were required to inhibit oleate activation ca. 70% when 2 μmol oleate were incubated with 400 μg mitochondrial protein and 13.7 mg albumin compared to approximately the same inhibition obtained with only 1 μmol retinoate, 636 μg microsomal protein, and 2 μmol oleate in the absence of albumin (Fig. 2). The inhibition was dependent on inhibitor concentration, fatty acid concentration, and configuration, and type and amount of protein included in the incubation mixture. At low retinoate concentrations (Fig. 2) little or no inhibition of palmitate activation was observed. At higher concentrations, retinoate also inhibited palmitate activation but to a much lower degree than it inhibited oleate activation. With 4 μmol retinoate and 804 μg mitochondrial protein, the degree of inhibition of palmitate activation remained constant and could not be overcome by addition of more plamitate to the incubation mixture (Fig. 4). Inhibition of *cis*-acid activation by retinoate, d-α-tocopheryl acid succinate or dihomogeranoate varied from one cell preparation to another and, depending on the structure of the acid, was usually 50-80% maximum when incubated with 500-800 μg microsomal or mitochondrial protein and 2 μmol fatty acid. Rapid inhibition of activation was observed at low concentrations of added retinoate, and a slower inhibition rate was observed as the retinoate concentration was increased.

TABLE I

Optimal Fatty Acid-Protein Ratios Required for Maximum Activation of Unsaturated Acids and Palmitate by Rat Liver Microsomes and Mitochondria[a]

Acid	Microsomes	Mitochondria
Oleate	4.2:1	1.8:1
Elaidate	8.4:1	3.6:1
cis-Vaccenate	4.2:1	1.8:1
trans-Vaccenate	8.4:1	7.0:1
Linoleate	4.2:1	1.8:1
Linelaidate	4.2:1	3.6:1
Palmitate	8.4:1	8.4:1

[a]Ratios that resulted in maximum formation of acyl hydroxamate for the fatty acid and cell fraction indicated are expressed in terms of μmoles fatty acid per milligram protein.

Other Detergents

In the presence of 840 μg mitochondrial protein, a concentration of 0.04% Triton X-100 did not affect palmitate activation over a concentration range of 0-4 mM fatty acid. Under these conditions, oleate activation was

TABLE II

Effect of Albumin on Acyl-CoA Formation of *cis-trans* Acids and Palmitate[a]

Experiment	Acid	Albumin, mg 0	1.3	2.6	4.9	19.5
1	Oleate	100(1.10)	107	108	101	47
	Elaidate	100(0.30)	305	315	386	180
	cis-Vaccenate	100(1.11)	114	121	109	48
	trans-Vaccenate	100(0.81)	163	175	156	94
	Linoleate	100(0.91)	130	131	134	66
	Linelaidate	100(0.56)	206	222	206	88
	Palmitate	100(0.53)	199	241	237	103
2	Oleate	100(0.70)	103	120	110	39
	Elaidate	100(0.50)	147	108	124	49
	cis-Vaccenate	100(0.81)	104	113	102	40
	trans-Vaccenate	100(0.77)	130	147	135	60
	Linoleate	100(0.27)	215	333	398	268
	Linelaidate	100(0.67)	164	145	144	57
	Palmitate	100(0.94)	132	133	103	108

[a]The indicated milligram of fatty acid free albumin were incubated with 2 μmol fatty acid potassium salt for 20 min at 37 C in a volume of 350 μl. After cooling to 4 C, the components of the standard incubation mixture plus 570 μg mitochondrial (Experiment 1) or 488 μg microsomal (Experiment 2) protein were added and the mixture was incubated for 30 min at 37 C. Results are expressed in per cent relative to the activation observed in the absence of albumin. Absolute values in μmoles hydroxamate formed are shown in parentheses.

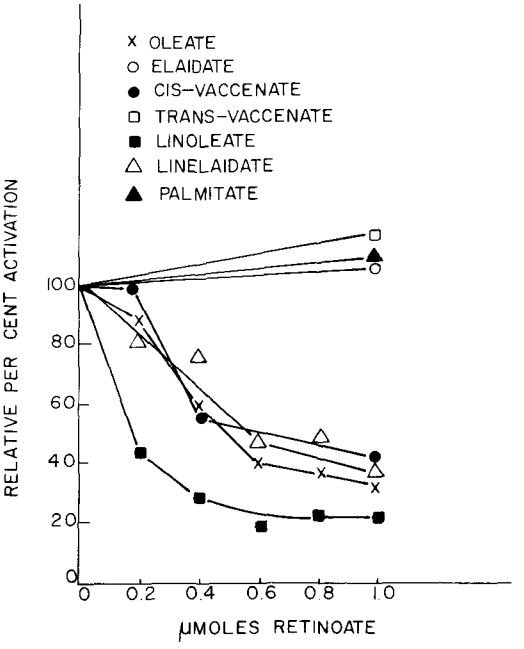

FIG. 2. Effect of retinoate on microsomal activation of *cis*- and *trans*-unsaturated acids and palmitate. Each tube contained the components of the standard incubation mixture plus 636 μg microsomal protein, 2 μmol fatty acid, the concentration of retinoate indicated in a final volume of 1.0 ml. Incubation was for 30 min at 37 C. Activation is expressed as μmoles hydroxamate formed relative to the activation in the absence of retinoate. The 100% values were: (X), oleate, 1.11; (○), elaidate, 1.31; (●), *cis*-vaccenate, 1.15; (□), *trans*-vaccenate, 1.45; (■), linoleate, 1.20; (△), linelaidate, 1.37; and (▲), palmitate, 1.58.

not affected by Triton X-100 in the presence of 0-1.6 mM oleate. However Triton X-100 markedly stimulated oleate activation at 1.6-4.0 mM oleate. These higher oleate concentrations were self-inhibitory in the absence of detergent. In contrast, the activation of elaidate was not affected by Triton X-100 in the presence of 0-2.5 mM fatty acid, while a slight stimulation was observed at higher elaidate concentrations. These results are shown in Figure 5. Similar results were observed with sodium deoxycholate. In contrast, with 636 μg microsomal protein as the enzyme source and in the presence of 2 μmol fatty acid and 0.1% Triton X-100, the activation of oleate was inhibited 91%, but the activation of palmitate and elaidate was only inhibited 27 and 34%, respectively. Under these conditions, 0.1% sodium lauryl sulfate completely inhibited palmitate, oleate and elaidate activation. At low concentrations, sodium deoxycholate stimulated oleate activation slightly but had no effect on palmitate or elaidate activation, while at higher concnetrations this detergent inhibited activation of all of these acids equally. At low concentrations, cetyl pyridinium chloride did not affect elaidate activation, slightly inhibited palmitate activation, and stimulated oleate activation 84%. At higher detergent concentrations, activation of all acids was inhibited. These results are summarized in Table III.

DISCUSSION

Fatty Acid-Protein Ratio

Previous investigators have noted that high

TABLE III

Effect of Detergents on Activation of Fatty Acids[a]

Acid	Relative per cent activation				
	d-α-Tocopheryl acid succinate, mM				
	0	0.13	0.25	0.50	
Palmitate	100(1.48)	—	—	105	
Elaidate	100(1.28)	102	—	95	
cis-Vaccenate	100(0.87)	62	41	—	
trans-Vaccenate	100(1.30)	—	103	102	
Linoleate	100(0.57)	37	39	39	
Linelaidate	100(0.69)	88	100	97	
Linolenate	100(1.01)	98	62	39	
Arachidonate	100(0.24)	67	69	75	
	Dihomogeranoate, mM				
	0	1	2	3	4
Palmitate	100(1.44)	103	94	88	77
Oleate	100(0.79)	38	29	15	6
	Triton X-100, %				
	0	0.1			
Palmitate	100(1.85)	73			
Oleate	100(1.13)	9			
Elaidate	100(1.53)	66			
	Sodium deoxycholate, %				
	0	.025	.050	.075	
Palmitate	100(1.85)	85	53	24	
Oleate	100(1.13)	122	87	22	
Elaidate	100(1.53)	108	82	21	
	Sodium lauryl sulfate, %				
	0	.025	.050	.100	
Palmitate	100(1.62)	18	0	0	
Oleate	100(0.64)	12	0	0	
Elaidate	100(1.28)	19	0	0	
	Cetyl pyridimium chloride, %				
	0	.025	.050	.100	
Palmitate	100(1.62)	80	52	0	
Oleate	100(0.64)	184	151	15	
Elaidate	100(1.28)	103	76	5	

[a]Each tube contained the components of the standard incubation mixture plus 2 μmol fatty acid, the concentration of detergent indicated, and 636-768 μg microsomal protein in a final volume of 1.0 ml. Incubations were for 30 min at 37 C. Results are expressed in terms of per cent activation relative to the activation in the absence of detergent. The absolute values for activation (μmol hydroxamate formed) in the absence of detergent are shown in parentheses.

concentrations of *cis*-unsaturated fatty acids inhibit acyl-CoA formation (27,29) and that reproducible results for K_M and V_{Max} for saturated fatty acids cannot be obtained due to the low solubility of these acids (30). In the present investigation, elaidate and *trans*-vaccenate did not inhibit acyl-CoA formation at concentrations at which the corresponding cis unsaturated acids oleate and *cis*-vaccenate almost completely inhibited their own activation. Possibly there is one rat liver long chain acyl-CoA synthetase with a catalytic site that binds saturated and unsaturated acids and an inhibitor site that only binds *cis*-unsaturated acids. If binding to the inhibitor binding site only occurred at *cis*-acid concentrations above the optimal fatty acid-protein saturation ratios, the inhibition of *cis*-acid activation observed here would be understandable.

Albumin stimulated activation of *cis*-mono- and -diunsaturated acids and of linelaidate, presumably by binding excess fatty acid (27) that caused inhibition of acyl-CoA synthetase in the absence of albumin. The variation in binding affinities of fatty acids with either *cis*- or *trans*-configurations to albumin remain to be elucidated.

Effect of *cis-trans* Isomerism

It is well established that the *trans*-unsaturated acids are generally metabolized in the same manner as saturated fatty acids, rather than as *cis*-unsaturated acids. The *trans*-isomers are incorporated into the 1 position of triglycerides and lecithin (2) and also resemble stearic acid rather than *cis*-unsaturated acids in their

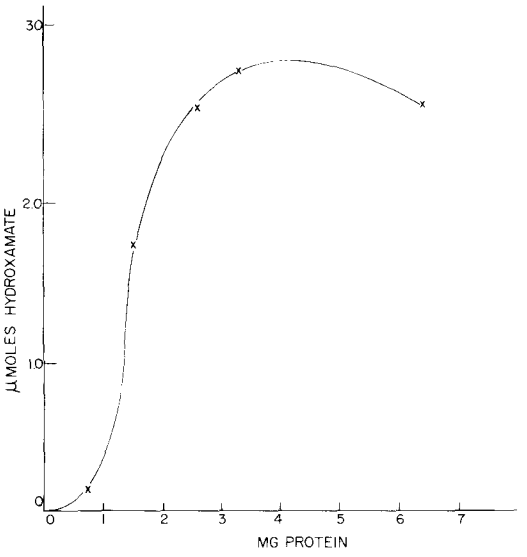

FIG. 3. Effect of mitochondrial protein on oleate activation in the presence of retinoate. Each tube contained the components of the standard incubation mixture plus 2 μmol oleate and 2 μmol retinoate and the milligram mitochondrial protein indicated in a total volume of 1.0 ml. Incubation was for 30 min at 37 C. Control tubes lacking ATP and coenzyme A were run for each level of protein tested. Results are expressed as μmoles hydroxamate formed per hour.

slow rate of cholesterol ester formation by rat liver microsomes (3). Similarly, in the present study, several properties of the *trans*-acids resembled those of the saturated acids rather than those of the *cis*-unsaturated acids, including optimal fatty acid-protein ratios required for maximum acyl-CoA formation, and activation insensitive to inhibition by low concentrations of isoprenoid acids.

The similar behavior in solution of *trans*- and saturated acids, as opposed to that of the *cis*-isomers described in this study, presumably is a reflection of the straight cylindrical configuration of the *trans*- and saturated acids as distinct from the L-shaped configuration of *cis*-unsaturated acids (31).

Effect of Positional Isomerism

Double bond position is quite important in some metabolic reactions. Acyl-coenzyme A-cholesterol-O-acyltransferase is highly specific for a single *cis*-double bond at a proximal distance of nine carbon atoms from the carboxyl group (3). Similarly, sterol ester hydrolase exhibits a preference for 9-octadecenoate, and activity decreases with movement of the ethylene bond to either end of the carbon chain (4,32). Acyl-CoA GPC transferase activities also show preference for specific positional isomers (33-35). In the present study, displacing the double bond from carbon 6 to carbon 11 of the *cis*-isomers, did not markedly affect acyl-CoA formation, since little difference was noted in the activation rates of petroselenate (Δ^6 octadecenoate) oleate (Δ^9 *cis*-octadecenoate) and *cis*-vaccenate (Δ^{11} *cis*-octadecenaote). Also, activation of the Δ^9 *trans*-acids was slightly lower than that of the Δ^{11} *trans*-acid. A more complete sutdy on the effect of double bond positional isomerism on the rate of acyl-CoA formation of *cis*- and *trans*-acids is discussed in the following paper.

Effects of Detergents on Acyl-CoA Formation

Isoprenoid acids: The inhibition of *cis*-acid acyl-CoA synthesis, but not of *trans*-acid or palmitate activation, by all-*trans* retinoate, dihomogeranoate and d-α-tocopheryl acid succinate could be interpreted as indicative of the presence of two activating enzymes in rat liver microsomes and mitochondria. However, due to the insolubility of the fatty acid substrates and the unknown interactions that occur between the inhibitor acids, enzyme and fatty acid, no definitive conclusion can be drawn. The observed inhibitions may represent solution interactions between the isoprenoid inhibitors and *cis*-acids, rather than inhibition of a distinct activating enzyme. Tocored, an oxidation product of tocopherol, forms an adduct at high temperatures with isomerized methyl linoleate but not with methyl palmitate (36). In view of the varied structures of the isoprenoid inhibitors used in the present study, formation of specific adducts with *cis*-acids, but not with *trans*- or saturated acids, does not seem probable. Since the inhibition was readily relieved by addition of extra microsomal or mitochondrial protein, the possibility of a nonspecific detergent effect seems more likely. However such an effect does not explain why the activation of *trans*-unsaturated acids is not inhibited also by low concentrations of these isoprenoid acid detergents. Further investigation is required to delineate the interactions of the isoprenoid acids, acyl-CoA synthetase and *cis*- and *trans*-unsaturated acids in greater detail.

Other detergents: Variable effects of detergents on acyl-CoA synthetase activity in different organisms have been reported by other investigators. Overath and coworkers reported a two-fold stimulation of an *E. Coli* mutant acyl-CoA synthetase when 0.01% Triton X-100 was included in the incubation medium, which included 1.3 mM oleate and an unspecified amount of enzyme (37). The authors indicated that they were uncertain whether the stimulation was due to the effect of the Triton on the

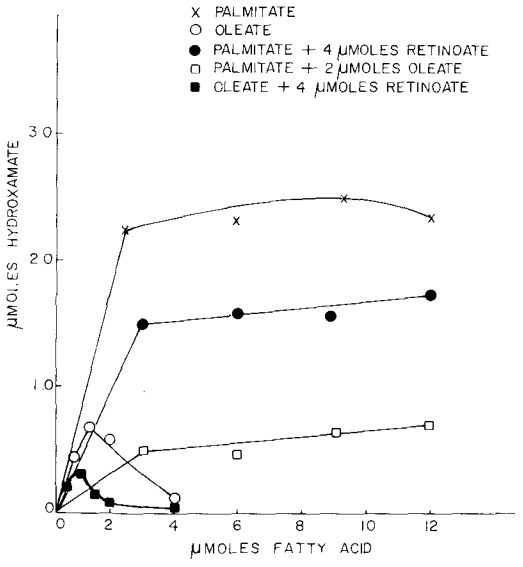

FIG. 4. Effect of retinoate and oleate on mitochondrial activation of palmitate and oleate. Each tube contained the components of the standard mixture, the concentrations of palmitate (X), oleate (○), and retinoate indicated plus 804 μg mitochondrial protein in a total volume of 1.0 ml. Incubation was for 30 min at 37 C. Results are expressed as μmoles hydroxamate formed.

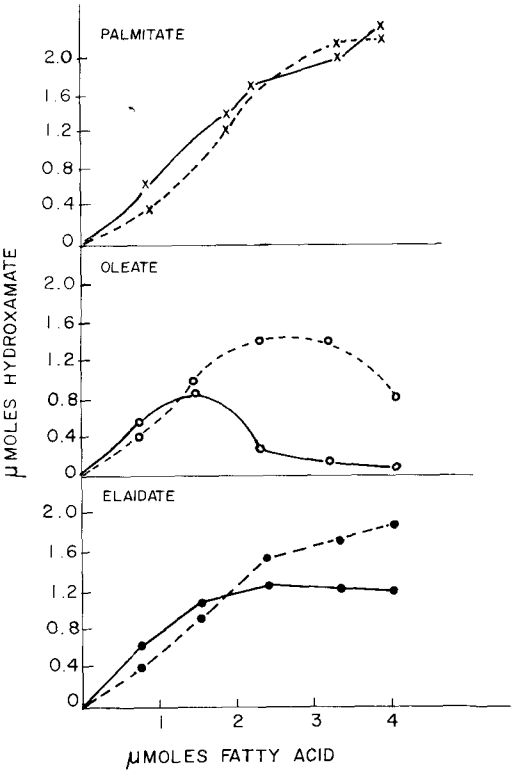

FIG. 5. Effect of Triton X-100 on mitochondrial activation of palmitate, oleate and elaidate. Each tube contained the components of the standard incubation mixture plus 840 μg mitochondrial protein, the μmoles palmitate (X), oleate (○), or elaidate (●) shown and 0.04% Triton X-100 where indicated (--) in a final volume of 1.0 ml. Incubation was for 30 min at 37 C. Results are expressed as μmoles hydroxamate formed.

enzyme or to solubilization of oleate by the detergent. Massoro and Lennarz reported that cetyl trimethylammonium bromide, sodium lauryl sulfate and Triton A-20 at 10^{-4} to 10^{-2}M and in the presence of 2.1 mM myristate and 150 μg *B. megaterium* protein had no stimulatory effect on acyl-CoA ester formation, while sodium lauryl sulfate inhibited 90% at 10^{-3}M and cetyltrimethylammonium bromide inhibited at greater than 10^{-3}M (38). Rat liver microsomal palmitate activation was reported to be 21% inhibited by 0.025% deoxycholate and 44% inhibited by 0.2% Triton X-100. The exact protein and fatty acid concentration were not specified, although the observation was made that Triton X-100 inhibition increased at lower protein levels and longer times of incubation (27). The data presented here show that the effect of any detergent depends on the fatty acid concentration, the fatty acid configuration, the amount and type of protein and the type and concentration of detergent included in the incubation medium. Apparent stimulation of oleate activation by Triton X-100 or sodium deoxycholate or cetyl pridinium chloride was observed at high oleate concentrations that are self-inhibitory in the absence of detergent. We attribute this apparent stimulation to incorporation of excess oleate into the detergent micelle. In this manner, the excess oleate may be prevented from inactivating acyl-CoA sysnthetase. Oleate activation was more than 90% inhibited by 0.1% Triton X-100, while palmitate or elaidate activation were only slightly inhibited (Table III). It is not clear, however, if this inhibition of oleate activation can be attributed to detergent inhibition of a distinct oleate activating enzyme or to selective incorporation of oleate but not elaidate or palmitate into the detergent micelle.

In studies with *E. Coli* mutants, based on two different lines of evidence, it was concluded that it was unlikely that the substrate specificity of the acyl-CoA synthetase is the major factor for the control of the fluidity, i.e., unsaturation of the membrane phospholipids (39). Similarly, the fatty acid configuration of complex lipids in mammalian tissues is presumably also determined by the specificity of the acyl-CoA transferases and not by acyl-CoA synthetase(s), since both saturated and unsatu-

rated fatty acids are incorporated into all positions of triglycerides and phospholipids in a manner that cannot be correlated with the rates of activation observed in this investigation.

The number of long chain fatty acid activating enzymes in rat liver is uncertain because the (enzyme or) enzymes are membrane-bound and have not been purified. Overath and coworkers concluded that the isolation of a single step mutant (old 88 from *E. Coli* which lacked the ability to activate fatty acids (C_6 to C_{20} *-cis-* or *trans*,- mono- or polyunsaturated) made it likely that only one enzyme was responsible for activation of all of these acids (37). Attempts to solubilize the rat liver activating enzyme(s) by several methods including freezing and thawing, sonication, acetone powder preparation and treatment with detergents were not successful (27). The possibility of two rat liver enzymes, one that activates saturated fatty acids and a second that activates *cis*-mono- and -polyunsaturated acids, has been suggested based on different coenzyme A requirements (27). The differences observed in this study in the requirements for maximum activation of *cis*-unsaturated vs. *trans*-unsaturated or -saturated fatty acids can be interpreted as support for the suggestion that two long chain activating enzymes are present in rat liver. However, in view of the insoluble nature of the fatty acid substrate and the membrane-bound enzyme(s) as well as the unknown manner of their interactions, the possiblility of a single long chain activating enzyme cannot be excluded.

A study of the properties of solubilized rat liver microsomal acyl-CoA synthetase published after the completion of this investigation lends support to the suggestion that a single enzyme activates both saturated and unsaturated long chain fatty acids (40).

The data presented here indicate that *trans*-unsaturated acids are treated enzymatically in a manner similar to saturated acids, rather than as *cis*-monounsaturated acids. The use of elaidic acid as a substitute for oleic acid in the production of experimental atheromas in rabbits (41) is therefore questionable.

REFERENCES

1. Sgoutas, D.S., and F.A. Kummerow, J. Clin. Nutr. 23:1111 (1970).
2. Lands, W.E.M., M.L. Blank, L.J. Nutter and D.S. Privett, Lipids 1:224 (1966).
3. Sgoutas, D.S., Biochem. 9:1826 (1970).
4. Goller, H.J., D.S. Sgoutas, I.A. Ismail and F.D. Gunstone, Biochem. 9:3072 (1970).
5. Sgoutas, D.S., Biochim. Biophys. Acta 164:317 (1968).
6. Bickerstaffe, R., and E.F. Annison, Biochem. J. 118:443 (1970).
7. Kleiman, R., F.R. Earle, W.H. Tallent, and I.A. Wolff, Lipids 5:513 (1970).
8. Filer, L.J., F.H. Mattson and S.J. Forman, J. Nutr. 99:293 (1969).
9. Anderson, R.L., Biochim. Biophys. Acta 152:531 (1968).
10. Anderson, R.L., Ibid. 144:18 (1967).
11. Anderson, R.L., and R.H. Coots, Ibid. 144:525 (1967).
12. Decker, W.J., and W. Mertz, J. Nutr. 89:165 (1966).
13. Struijk, C.B., and R.K. Beerthius, Biochim. Biophys. Acta 116:12 (1966).
14. Privett, O.S., L.J. Nutter and F.S. Lightly, J. Nutr. 89:257 (1966).
15. Selinger, Z., and R.T. Holman, Biochim. Biophys. Acta, 106:56 (1965).
16. Knipperath, W.G., and J.F. Mead, JAOCS 41:437 (1964).
17. Jensen, R.G., J. Sampugna and R.L. Pereira, Biochim. Biophys. Acta 84:481 (1964).
18. Coots, R.H., J. Lipid Res. 5:468 (1964).
19. Privett, O.S., and M.L. Blank, JAOCS 41:292 (1964).
20. Ono, K., and D.S. Frederickson, J. Biol. Chem. 239:2482 (1964).
21. Coots, R.H., J. Lipid Res. 5:473 (1964).
22. Lands, W.E.M., JAOCS 41:46 (1964).
23. Blank, M.L., and O.S. Privett, J. Lipid Res. 4:470 (1963).
24. Raulin, J., C. Loriette and G. Clement, Biochim. Biophys. Acta 70:642 (1963).
25. Lippel, K., JAOCS 48 (7): Abstr. 148 (1971).
26. Schneider, W.C., and G.H. Hogeboom, J. Biol. Chem. 183:123 (1950).
27. Pande, S.V., and J.F. Mead, Ibid. 243:352 (1968).
28. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, Ibid. 193:265 (1951).
29. Kornberg, A., and W.E. Pricer, Ibid. 204:329 (1953).
30. Borgstrom, B., and L.W. Wheeldon, Biochim. Biophys. Acta 50:171 (1961).
31. Vandenheuvel, F.A., Ibid. 40:455 (1963).
32. Goller, H.J., and D.S. Sgoutas, Biochem. 9:4801 (1970).
33. Reitz, R.C., W.E.M. Lands, W.C. Christie and R.T. Holman, J. Biol. Chem. 243:2241 (1968).
34. Reitz, R.C., M. El-Sheikh, W.E.M. Lands, I.A. Ismail and F.D. Gunstone, Biochim. Biophys. Acta 176:480 (1969).
35. Jezyk, P., and W.E.M. Lands, J. Lipid Res. 9:525 (1968).
36. Komoda, M., and I. Harada, JAOCS 47:249 (1970).
37. Overath, P., G. Pauli and H.U. Schairer, Eur. J. Biochem. 7:559 (1969).
38. Massoro, E.J., and W.J. Lennarz, Biochem. 4:85 (1965).
39. Esfahani, M., T. Ioneda and S.J. Wakil, J. Biol. Chem. 246:50 (1971).
40. Bar-Tana, J., G. Rose and B. Shaprio, J. Biochem. 122:353 (1971).
41. Dayton, S., and S. Hashimoto, J. Ather. Res. 8:555 (1968).

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Activation of Long Chain Fatty Acids by Subcellular Fractions of Rat Liver: II. Effect of Ethylenic Bond Position on Acyl-CoA Formation of *trans*-Octadecenoates

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ABSTRACT

The rates of acyl-CoA biosynthesis of the *trans* Δ^4 to Δ^{15} octadecenoates have been studied using rat liver microsomes and mitochondria as the enzyme(s) source. Acyl-CoA formation was lowest when the Δ^9 isomer was the substrate and increased as the double bond was moved to either end of the fatty acid chain. Maximal activation was observed when the Δ^6 and Δ^{14} *trans*-octadecenoates were substrates. All-*trans* retinoate caused differential inhibition in the activation rates of the various *trans*-octadecenoate isomers. In contrast, the activation profile relative to double bond position was not altered by changing the incubation temperature, pH, or buffer or by adding fatty acid free albumin to the incubation medium or by aging the enzyme. These results do not support the suggestion that more than one enzyme activates the various *trans*-octadecenoate positional isomers. Triton X-100 differentially activated acyl-CoA biosynthesis of the various isomers resulting in a uniform rate of activation. Release of the isomers from the detergent micelle is suggested to be rate-limiting under these conditions.

INTRODUCTION

In the preceding paper the conditions required for maximum activation of *trans*-vaccenate and elaidate were found to be similar to the conditions required for maximum activation of saturated acids, but distinct from those for optimal activation for *cis*-acids (1). Also, slight differences were observed in the rates of activation of the Δ^9 and Δ^{11} *trans*-acids, elaidate and *trans*-vaccenate. The present study was undertaken to analyze in greater detail the effect of the double bond position on the activation of *trans*-octadecenoates by rat liver subcellular fractions.

EXPERIMENTAL PROCEDURES

Materials

trans-Octadecenoates were synthesized as described previously (2). ATP, coenzyme A and

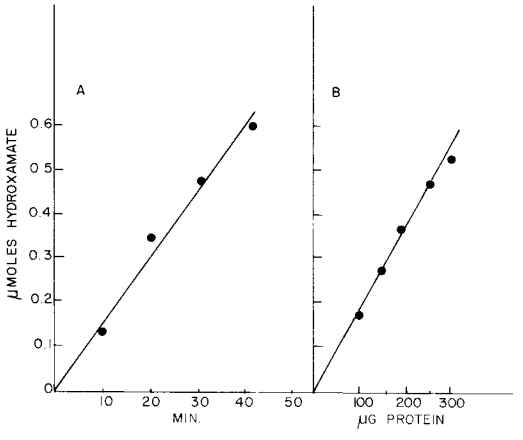

FIG. 1. Effect of incubation time and protein concentration on activation of *trans*-vaccenate. Each tube contained the components of the standard incubation mixture plus 245 μg (A) or the micrograms of mitochondrial protein indicated (B). Incubation was at 37 C for 30 min (right side) or for the times shown (left side).

TABLE I

Effect of Fatty Acid Concentration on Microsomal Acyl-CoA Formation of Positional *trans*-Isomeric Octadecenoates[a]

Position of *trans*-double bond	Fatty acid concentration, mM			
	1.6	3.2	4.0	8.0
4	2.33	4.46	4.45	4.63
5	2.17	4.16	4.65	4.37
6	2.04	4.73	5.52	4.91
7	1.93	3.91	4.43	3.17
9	1.78	3.70	2.86	1.86
10	2.20	4.24	4.08	3.50
11	2.13	3.89	3.83	3.24
12	2.03	4.05	4.74	3.77
13	2.35	4.15	4.70	3.89
14	2.05	4.64	5.11	5.60
15	2.07	4.88	4.63	4.41

[a]Each tube contained the components of the standard incubation mixture and the concentrations of fatty acid shown. Incubation was for 30 min at 37 C. Results are expressed as μmoles hydroxamate formed per hour per milligram protein.

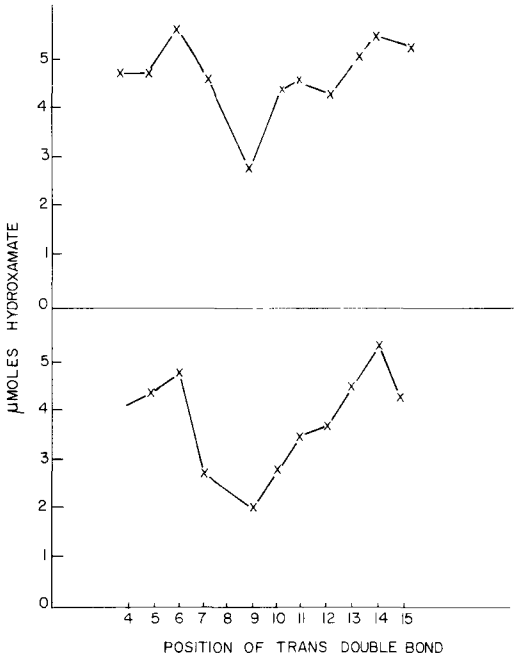

FIG. 2. Effect of double bond position on the rates of microsomal and mitochondrial activation of *trans*-octadecenoates. Each tube contained the components of the standard incubation mixture plus 205 μg of microsomal or mitochondrial protein in a total volume of 0.25 ml. Incubation was for 30 min at 37 C. Results are expressed as μmoles of hydroxamate formed per milligram protein per hour for microsomes (A) and mitochondria (B).

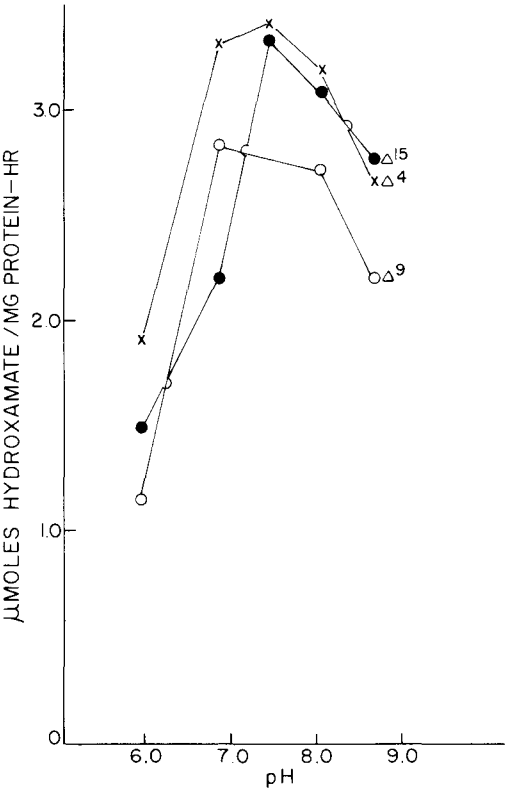

FIG. 3. Effect of pH on mitochondrial activation of *trans*-octadecenoates. Each tube contained the components of the standard incubation mixture plus 205 μg mitochondrial protein and either 0.1 M phosphate buffer (pH 6.0 to pH 8.0) or 0.1 M Tris-HCl buffer (pH 8.6). Results shown are for the Δ^4, (X); Δ^9, (○); and the Δ^{15}, (●) *trans*-octadecenoates.

Triton X-100 (polyoxyethylene alcohol lauryl ether) were from Sigma Chemical Co. Fatty acid free albumin fraction V was from Pentex Biochemicals, Kankakee, Ill. All-*trans* retinoate was purchased from Distillation Products Industries, Rochester, N.Y.

Methods

White Wistar strain rats of either sex (Microbiological Associates) were used in all experiments. The rats were 6-10 weeks of age and had been fed ad libitum on Purina rat pellets. Cell fractions were prepared as described previously (1). Acyl-CoA formation was assayed by acyl-hydroxamate formation (3). The standard incubation mixture contained the following μmoles in a volume of 250 μl: a mixture of H_2NOH, 125; NaF, 6; Tris (Tris [hydroxymethyl] aminomethane), 25; neutralized to pH 7.4; magnesium chloride, 1; cysteine HCl, 7.5 freshly neutralized with Tris, 10; ATP, 2.5; coenzyme A, 0.125; fatty acid potassium salt, 1.0; and ca. 200 μg mitochondrial or microsomal protein. Incubation was for 30 min at 37 C on a Dubnoff shaker. The reaction was ended by addition of 1.0 ml of 6% perchloric acid, and after centrifugation the supernatant was discarded. Acyl-hydroxamic acid was extracted with 1.2 ml Hill reagent A diluted 1:10 with ethanol, and after centrifugation the optical density at 520 nm was read in a Beckman-DU spectrophotometer. Protein was determined by the phenol method (4).

RESULTS

Activation of the *trans*-octadecenoate positional isomers was proportional to the amount of protein and incubation times up to 40 min as illustrated in Figure 1 for *trans*-vaccenate. As shown in Table I, activation increased with fatty acid concentrations up to 4 mM and then decreased slightly at higher concentrations. Maximum activation with both microsomes and mitochondrial acyl-CoA synthetases was observed with the Δ^6 and Δ^{14} *trans*-octadecenoates. Activation decreased as the double bond

was moved in from either end of the fatty acid toward the ninth carbon atom from the carboxyl group. These results are shown in Figure 2. The Δ^8 *trans*-octadecenoate was tested separately. In a typical experiment, the average rates of microsomal activation for an assay in triplicate for the Δ^7, Δ^8 and Δ^9 isomers, respectively, expressed as μmoles of hydroxamate formed per hour per milligram protein were: 5.09, 5.25, 4.60. Replicates were all within ±5% from the average value.

Addition of up to 1.25 mg fatty acid free albumin to the incubation medium did not markedly affect the activation of the *trans*-octadecenoates, although slight stimulation in the activation of the Δ^4, Δ^{11}, and Δ^{14} isomers (+22%, +33% and +20%) was observed upon the addition of albumin. The same general activation profile was observed in the presence or absence of albumin with minimum activation observed with the Δ^9 isomer and maximum activation observed with the Δ^6 and Δ^{14} isomers as substrates.

Effect of pH, Buffer and Temperature

Activation of each *trans*-isomer was similar over the pH range 6.7 to 8.0 and decreased as the pH was either increased to 8.6 or lowered to 6.0. The same general activation profile was observed as discussed previously, i.e., minimum activation was found when the Δ^9 isomer was the substrate and higher rates of activation were observed as the position of double bond was moved toward either end of the fatty acid. Similar activation profiles were obtained with Tris, phosphate, glycylglycine or barbital buffers at pH 7.4. Activation profiles for the Δ^4, Δ^9 and Δ^{15} isomers at various pH values are illustrated in Figure 3. The same activation profile was observed at 22 C, 30 C, 37 C and 44 C, i.e., maximum activation with the Δ^5, Δ^6 and Δ^{14} isomers and minimum activation when the Δ^9 isomer was the substrate. Activity was optimal at 37 C and decreased two-fold at 30 C and four- to ten-fold at 44 C.

Effect of Aging of Cell Fractions on Acyl-CoA Synthetase Activity

Borgstrom and Wheeldon found guinea pig liver long chain acyl-CoA synthetase to be highly labile, i.e., oleate activation declined ca. 50% after 18 hr at 4 C and 80% after 1 hr incubation at 37 C (5). In contrast, Creasey found no loss of microsomal acyl-CoA synthetase after 2 weeks storage at -18 C (6); similar stability of the rat liver microsomal enzyme at -20 C was noted in the present study, i.e., microsomal activation of the *trans*-octadecenoate was not affected by storage of the microsomes at -20 C for 10 days. Activity declined slightly after 20 days storage at -20 C, but the same activation profile was retained (Table II).

TABLE II

Effect of Aging on Microsomal Activation of *trans*-Octadecenoates[a]

Position of *trans*-double bond	Age of fraction when tested, days		
	1	10	20
4	4.65	4.48	3.63
5	4.65	4.54	3.74
6	5.50	5.27	4.12
7	4.12	4.10	3.42
9	2.81	3.10	2.71
10	4.27	4.51	3.76
11	4.37	3.95	3.56
12	4.15	4.37	3.71
13	4.97	4.70	4.09
14	5.34	4.93	4.22
15	4.80	4.59	3.92

[a]Each tube contained the components of the standard incubation mixture plus 205 μg microsomal protein in a total volume of 250 μl. Results are expressed in μmoles hydroxamate per milligram protein per hour. Cell fractions were stored at -20 C.

Effect of Detergents

In the preceding study it was found that 1 mM retinoate did not affect activation of 2 mM elaidate or 2 mM *trans*-vaccenate in the presence of 636 μg microsomal or mitochondrial protein. In this study addition of 1.2 mM retinoate inhibited activation of 4 mM Δ^9 and Δ^{12} octadecenoates 30-50% and 23-37%, respectively, in the presence of 205 μg microsomal or mitochondrial protein. Variable inhibition of the Δ^5 isomer was also noted, and the other positional isomers were only slightly inhibited. Addition of 0.04% Triton X-100 differentially stimulated mitochondrial activation 3-60%, so that the activation observed with the *trans*-octadecenoate isomers all fell in the same range. These results are summarized in Table III.

DISCUSSION

In the present study, lower rates of activation were observed when the double bond was localized at carbons 9 or 10, than when the double bond was positioned at either end of the fatty acid molecule.

Similar results were observed with *cis*-octadecenoate positional isomers (7). These results together suggest the possibility that one important factor in enzyme recognition of the positional isomeric octadecenoates is the length of nine carbon atoms from the carboxyl group to

TABLE III

Effect of Retinoate and Triton X-100 on Activation of *trans*-Octadecenoates[a]

Position of *trans*-double bond	Microsomes			Mitochondria				
	Detergent added			Detergent added				
	None	Retinoate	Δ, %	None	Retinoate	Δ, %	Triton X-100	Δ, %
4	4.48	4.53	+ 1	2.22	2.30	+ 4	3.17	+43
5	4.54	4.09	-10	3.11	1.85	-41	3.19	+ 3
6	5.27	4.44	-16	3.22	2.59	-20	3.57	+11
7	4.10	3.40	-17	2.20	1.73	-22	3.54	+61
9	3.10	2.13	-31	2.39	1.17	-51	2.94	+23
10	4.51	3.41	-24	2.33	2.05	-12	3.41	+46
11	3.95	3.91	- 1	2.56	2.22	-13	3.13	+22
12	4.37	3.37	-23	2.52	1.60	-37	3.47	+38
13	4.70	4.29	- 9	3.27	2.76	-16	3.37	+ 3
14	4.93	5.07	+ 3	3.40	2.97	-13	3.38	+14
15	4.59	4.48	- 2	3.00	2.34	-22	3.51	+17

[a]Each tube contained the components of the standard incubation mixture plus either 205 μg microsomal or mitochondrial protein and where indicated 0.3 μmol potassium retinoate or 0.04% Triton X-100 in a total volume of 250 μl. Incubation was for 30 min at 37 C. Results are expressed as μmoles hydroxamate formed per milligram protein per hour, and the change in activity upon addition of detergent (Δ) is expressed as the per cent change in activity relative to the activity in the absence of detergent.

the double bond and that the configuration of the double bond is of secondary importance. Furthermore the fatty acid to protein ratios required for maximum activation were much lower when the Δ^9, Δ^{10} or Δ^{15} isomers served as substrates, than when the other isomers were used as substrates. As shown in Table I, maximum activation of the Δ^9, Δ^{10} or Δ^{15} isomers occurred at 3.2 mM acid concentration, while activation of other isomers increased up to 4 mM acid concentration. In the previous paper (1), we found that the fatty acid-protein ratio required for maximum elaidate activation was much higher than the ratio required for maximum oleate activation. It is not clear from the present study whether these variations in fatty acid-protein ratios needed for optimal activation are related to enzyme specificity or are a reflection of different physical or solution properties of the various geometrical or positional isomers. The melting points of the *trans*-octadecenoates show alternation between odd and even number of fatty acid chain length (2) and cannot be correlated with relative activation rates. The solubility of both *cis*- and *trans*-octadecenoates in various solvents increases as the double bond is moved from carbon 6 to 9 (8). However no simple relationship between solubility and rate of activation is apparent from the results presented here, since increasing solubility with increasing distance of the double bond from the carboxyl group would result in an activation profile different from the activation profile reported here. Possibly the size and shape of the micelles formed by the various positional isomers may vary and result in different binding affinities for the catalytic site of the enzyme. The exact relationship between positional isomerism, micelle structure and rate of activation remains to be elucidated.

The differential inhibition of activation of the various positional isomers observed upon addition of retinoate suggests the possibility that more than one rat liver enzyme may activate the positional *trans*-octadecenoate isomers. However the presence of one or more activating enzymes is difficult to determine from the present data in view of the crude cell fractions and the unknown fatty acid-enzyme interactions. Nor do the parallel changes in activation rates of the positional isomers observed in the present study under different conditions of temperature, pH and aging lend support to the suggestion that more than one enzyme activates the *trans*-octadecenoate isomers. The retinoate inhibition of elaidate activation observed in this study (Table III) but not noted in the previous paper (Fig. 2 of Part I) is probably due to the much higher (fatty acid + retinoate to protein) ratio used in this study, i.e., (26 μmol/mg protein) compared to a lower ratio of (4.5 μmol/mg protein) used in the former study. When Triton X-100 was included in the incubation medium, activation of the octadecenoate positional isomers all fell in the same range, in contrast to widely varying activation rates in the absence of this detergent. Possibly the fatty acid isomers are incorporated into the detergent micelle. Release of fatty acid from the micelle may be rate-limiting under these conditions. The differential rates of acti-

vation observed in the present study may not reflect the rates at which the positional isomers are incorporated into lipids. Very little difference was observed in the rates of incorporation of the Δ^9 and Δ^{10} *trans*-octadecenoates into liver lipids using a perfused chicken liver system (9). More complete studies on the relationship of *trans*-double bond position and rates of incorporation of these positional octadecenoate isomers into complex lipids are required.

REFERENCES

1. Lippel, K., Lipids 8:00 (1973).
2. Barve, J.A., and F.D. Gunstone, Chem. Phys. Lipids 7:311 (1971).
3. Pande, S.F., and J.F. Mead, J. Biol. Chem. 243:352 (1968).
4. Lowry, D.H., N.G. Rosenbrough, A.L. Farr and R.G. Randall, J. Biol. Chem. 193:265 (1951).
5. Borgstrom, B., and L.W. Wheeldon, Biochim. Biophys. Acta 50:171 (1961).
6. Creasey, W.A., Ibid. 64:559 (1952).
7. Lippel, K., D. Carpenter, F.D. Gunstone and I.A. Ismail, Lipids 8:00 (1973).
8. Kolb, D.K., and J.B. Brown, JAOCS 32:357 (1955).
9. Bickerstaffe, R., and E.F. Annison, Biochem. J. 118:433 (1970).

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Activation of Long Chain Fatty Acids by Subcellular Fractions of Rat Liver: III. Effect of Ethylenic Bond Position on Acyl-CoA Formation of *cis*-Octadecenoates

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ABSTRACT

The rates of rat liver microsomal and mitochondrial activation of the Δ^4 to Δ^{17} *cis*-octadecenoate positional isomers have been investigated. The fatty acid to protein ratios required for maximum activation of the Δ^8, Δ^9 and Δ^{10} isomers were much lower than the corresponding ratios required for maximum activation of the *cis*-octadecenoates with double bonds at either end of the fatty acid molecule. Also, as the incubation temperature was raised from 22-38 C the Δ^8 and Δ^9 isomers exhibited little change in their rates of activation, while large increases in activation rates of the isomers with the double bond at either end of the fatty acid chain were observed. Differential inhibition of the activation of the various positonal isomers was observed when anionic, cationic, or nonionic detergents were included in the incubation medium. The different responses to fatty acid concentration, temperature and detergents are attributed to enzyme specificity and to differences in solution properties of the *cis*-octadecenoates, rather than to the presence of separate rat liver enzymes that catalyze acyl-CoA ester formation of the various positional isomers

INTRODUCTION

In previous investigations, the parameters affecting activation of *trans*-octadecenoates and the effect of positional isomerism on the activation of these acids were studied (1,2). The naturally occurring octadecenoates have the *cis* configuration and the predominant isomer in plant and animal lipids is the Δ^9 isomer oleate. Small percentages of other positional isomers also occur in mammalian lipids through ingestion of fish and other lower species. The purpose of the present study was to determine how positional isomerism affects rat liver acyl-coenzyme A biosynthesis of the *cis*-octadecenoates.

MATERIALS AND METHODS

Methyl esters of the *cis*-octadecenoates were synthesized as described previously (3). ATP, coenzyme A and Triton X-100 (polyoxyethylene alcohol lauryl ether) were from Sigma Chemical. Fatty acid free albumin fraction V was from Pentek Biochemicals, Kankakee, Ill. All-*trans* retinoate was purchased from Distillation Products Industries, Rochester, N.Y.

The methods used for preparation of rat liver cell fractions and assay of acyl-CoA formation by acyl-hydroxamate formation were the same as described in the previous paper of this series (2). The standard incubation mixture contained the following μmoles in a volume of 250 μl: a mixture of hydroxylamine HCl, 125; NaF, 6.25; Tris, 25 neutralized to pH 7.4; $MgCl_2$, 1.0; cysteine, HCl, 7.5 neutralized with Tris, 10; ATP, 2.5; coenzyme A, 0.13; fatty acid potassium salt, 0.5; and 200-250 μg microsomal or mitochondrial protein. Incubation was for 30 min at 37 C and acyl-hydroxamate formation was assayed at 520 nm in a Beckmann DU-2 spectrophotometer. Control tubes lacking ATP and coenzyme A were run with each assay. Protein was determined by the phenol method (4).

Methyl esters were converted to free acids by saponification in 5% KOH in 90% methanol for 2 hr at 70 C. After acidification with HCl, the free acids were extracted with petroleum ether. The extracts were washed with water and dried by passing through a small sodium sulfate column.

RESULTS

Effect of Fatty Acid Concentration

In the presence of 200 μg microsomal protein and nonsaturating levels of fatty acid (1 mM), similar rates of microsomal acyl-CoA formation, i.e., 105 ± 0.25 μmol hydroxamate formed per hour per milligram protein were observed, regardless of the position of the double bond in the fatty acid molecule. Similarly, at 2 mM, the optimal concentration of fatty acid for maximum activation of the Δ^8, Δ^9 and Δ^{10} isomers, the rates of activation of all the isomers were nearly equal. At a fatty acid concentration of 3 mM, the activation of the Δ^8, Δ^9 and Δ^{10} isomers decreased while activation of the other positional isomers in-

creased. A two- to three-fold difference between the rates of activation of the Δ^8, Δ^9 and Δ^{10} isomers and the rates of activation of the isomers with the double bond at either end of the fatty acid chain resulted. These differences in activation rates were further magnified at a still higher fatty acid concentration, i.e., an eight-fold difference between the lowest activation rate (Δ^8 isomer), and the highest activation rate (Δ^{16} isomer) was observed at a fatty acid concentration of 4 mM. Similar results were obtained with the mitochondrial acyl-CoA synthetase. These results are illustrated in Figure 1 and summarized in Table I. In the presence of 2 mM acid, increasing the microsomal protein from 200 to 400 μg resulted in little or no increase in the rates of activation above the levels observed using 200 μg protein.

Effect of Retinoate and Detergents

Retinoate inhibited the positional *cis*-octadecenoates in a differential manner. Maximum inhibition was observed when the double bond was at carbon atoms 6 to 10 and lower, or no inhibition was observed as the double bond was moved toward either end of the fatty acid molecule. In the presence of 1 mM retinoate, 78% inhibition of the activation of the Δ^8 isomer was noted, while the activation of the Δ^4 and Δ^5 isomers was 26% and activation of the Δ^{17} isomer was uninhibited. Addition of 0.05% of either Triton X-100 or sodium deoxycholate did not noticeably affect activation in the presence of 200 μg microsomal protein and 2 mM *cis*-octadecenoate. Addition of 0.05% of the cationic detergents cetyl pyridinium chloride caused differential inhibition of the microsomal activation which varied from a low of 3% for the Δ^9 isomer to a high of 43% for the Δ^{16} *cis*-octadecenoate. These results are summarized in Table II.

Effect of Albumin on Microsomal Activation

Addition of up to 1.0 mg fatty acid free bovine serum albumin to the incubation mixture containing 200 μg microsomal protein and 2 mM fatty acid did not affect the rate of microsomal activation of any of the *cis*-octadecenoates. The activation rates decreased (except the Δ^8 isomer) 3 to 29% when the added albumin was increased to 1.5 mg per tube.

Effect of pH on Microsomal Activation

Maximum microsomal activation of the *cis*-octadecenoates was observed over a broad pH range of 6.8 to 8.0, and the rates of activation decreased when the pH was lowered to 5.9 or raised to pH 8.6. The activation profile was

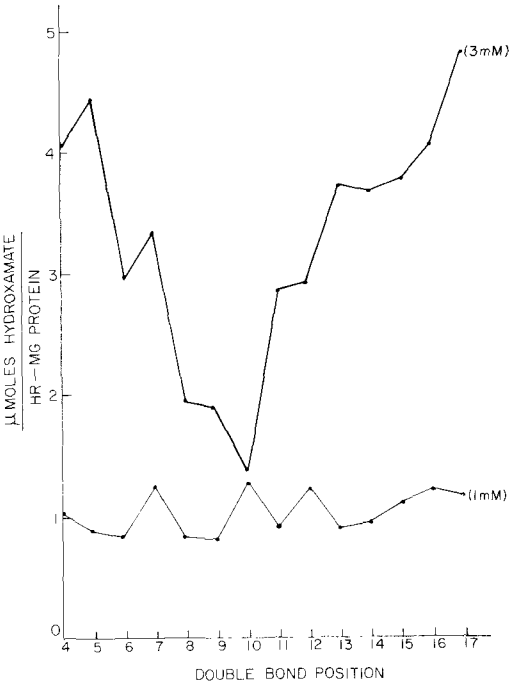

FIG. 1. Effect of fatty acid concentration on activation of *cis*-octadecenoate positional isomers. Incubation conditions are given in Table I.

approximately the same at each pH, i.e., higher rates of activation were noted when the double bond was at either end of the *cis*-octadecenoate molecule and relatively lower activation rates were observed for the *cis*-isomers with the double bond in the mddle of the fatty acid chain. Activation profiles for the Δ^4, Δ^9 and Δ^{15} isomers at various pH values are shown in Figure 2.

Effect of Temperature and Aging

Marked increases in mitochondrial activation of the Δ^4 to Δ^7 and Δ^{10} to Δ^{17} *cis*-octadecenoates were observed as the incubation temperature was increased from 22 C to 37 C. In contrast, less marked changes in the rates of activation were noted for the Δ^8 or Δ^9 isomers as the incubation temperature was raised. These results are illustrated in Figure 3. Aging the mitochondrial fraction at -20 C for 15 days resulted in partial loss of enzyme activity. However the same general activation profile was observed with both aged and fresh cell fractions.

DISCUSSION

The rates of activation of the positional isomeric *cis*-octadecenoates observed in this

TABLE I

Effect of Fatty Acid Concentration on Activation of *cis*-Octadecenoate Positional Isomers[a]

Position of double bond	Fatty acid concentration, mM							
	1	2	3	4	1	2	3	4
	Microsomes				Mitochondria			
4	1.03	2.86	4.06	4.73	1.44	2.64	3.22	2.11
5	0.88	2.83	4.44	4.91	1.36	2.58	1.53	0.52
6	0.84	2.34	2.94	1.20	0.92	1.40	0.24	0.06
7	1.26	2.62	3.34	2.00	1.28	2.09	0.28	0.23
8	0.84	2.21	1.94	0.70	0.77	0.44	0.24	0.11
9	0.82	2.38	1.90	0.79	0.89	0.52	0.26	0.26
10	1.28	2.28	1.38	0.84	1.01	0.53	0.23	0.33
11	0.90	2.35	2.87	1.25	1.04	1.21	0.39	0.23
12	1.24	2.77	2.94	1.22	0.86	1.41	0.46	0.28
13	0.94	2.62	3.73	3.68	1.12	2.20	0.77	0.66
14	0.97	2.32	3.68	4.43	1.03	2.12	2.96	1.64
15	1.13	2.89	3.70	4.73	1.07	2.13	2.97	2.99
16	1.24	3.07	4.06	5.42	1.14	2.71	3.32	3.67
17	1.19	2.80	4.51	4.81	1.06	2.08	3.12	3.13

[a]Each tube contained the components of the standard incubation mixture plus 200 μg microsomal or mitochondrial protein. Incubation was for 30 min at 37 C. Results are expressed as μmoles hydroxamate formed per hour per milligram protein.

investigation are not correlated with the natural occurrence of these isomers. On the contrary, at high concentrations (2-4 mM) the activation rate of the naturally occurring Δ^9 isomer, oleate, was the lowest or next to the lowest observed in comparison with the activation rates of the other isomers. However at a low concentration (1 mM) the activation rates for the various positional isomers all fell in the same range. At concentrations greater than 2 mM, olete (or the Δ^8 or Δ^{10} isomers) may bind to an inhibitory site and self-inhibit further activation. The physiological significance of the self-inhibition of activation observed here, using high concentrations of oleate, is dubious for several reasons. First, long chain acyl-CoA esters rather than free fatty acids accumulate during fasting (5). Fatty acid activation does not therefore appear to be a rate-limiting step in the utilization of extracellular fatty acids by

TABLE II

Effect of Retinoate and Detergents on Microsomal Activation of *cis*-Octadecenoates[a]

Position of double bond	Retinoate concentration, mM			Detergent			
	0	0.5	1.0	None	Triton X-100	Sodium deoxycholate	Cetyl pyridinium chloride
4	100(1.65)	82	74	100(2.20)	99	104	72
5	100(2.35)	111	74	100(2.46)	85	83	72
6	100(1.64)	62	30	100(1.82)	105	86	76
7	100(1.95)	83	24	100(2.11)	93	97	69
8	100(1.34)	41	22	100(1.93)	88	82	76
9	100(1.53)	62	30	100(1.79)	112	88	97
10	100(1.19)	50	27	100(1.97)	101	86	74
11	100(1.58)	81	53	100(2.22)	93	87	90
12	100(1.47)	64	64	100(1.58)	—[b]	—[b]	—[b]
13	100(1.82)	100	48	100(2.42)	138	92	74
14	100(1.85)	84	71	100(1.82)	97	111	80
15	100(1.99)	115	85	100(2.27)	91	100	79
16	100(2.10)	85	74	100(1.17)	77	103	57
17	100(1.73)	111	106	100(1.07)	85	96	73

[a]Each tube contained components of the standard incubation mixture plus 203 μg microsomal protein and 0.05% detergent or concentration of retinoate indicated. Results are given as per cent activation relative to activation observed in the absence of retinoate or detergent. Absolute activation values in μmoles hydroxamate formed per milligram protein per hour are shown in parentheses. Effects of detergents on the Δ^{16} and Δ^{17} octadecenoates were determined separately.

[b]Not tested.

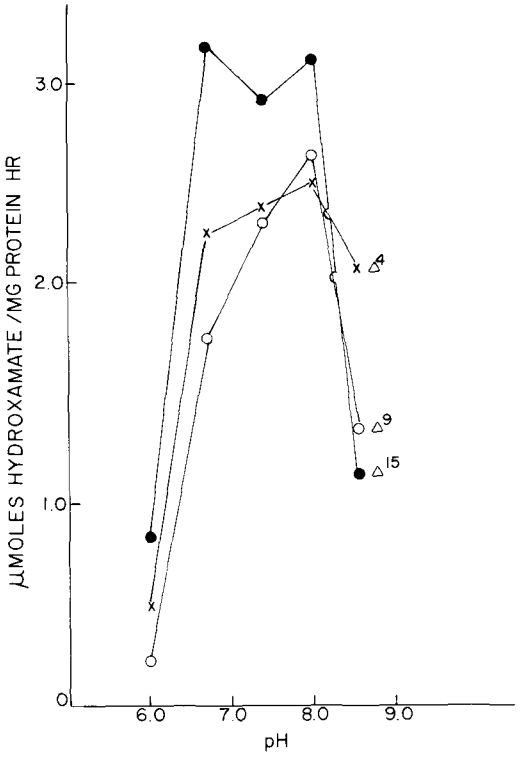

FIG. 2. Effect of pH on microsomal activation of *cis*-octadecenoates. Each tube contained components of the standard incubation mixture plus 200 μg microsomal protein and either 0.1 M phosphate buffer (pH 5.9 or pH 6.8) or 0.1 M Tris-HCl buffer (pH 7.4, pH 8.0 or pH 8.6) in a final volume of 250 μl. Results shown are for the Δ^4, (X); the Δ^9, (○); and the Δ^{15}, (⊙) octadecenoates.

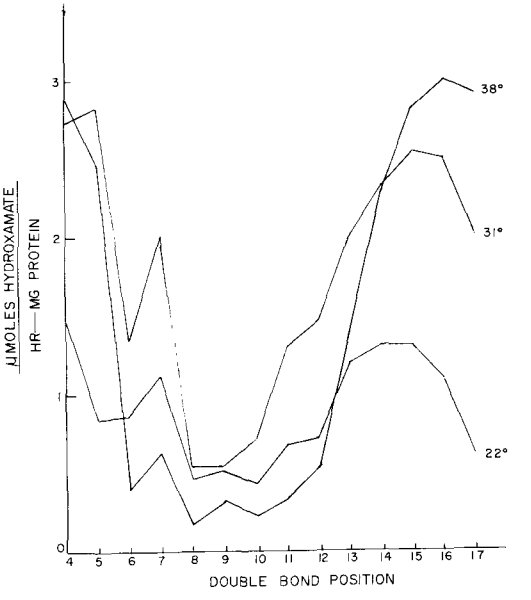

FIG. 3. Effect of temperature on activation of *cis*-octadecenoate positional isomers. Each tube contained components of standard incubation mixture plus 216 μg mitochondrial protein in a total volume of 250 μl.

the liver. Second, the intracellular level of liver free fatty acids remains constant at 1 μmol/g wet liver when the liver is perfused with greater than physiological plasma concentrations of oleic acid (6). The latter observation does not rule out the possibility of higher inhibitory concentrations of oleate accumulating in a specific cellular compartment. Third, in studies with isolated liver cells the estimated maximum lipid removal rate from the medium for a 10 g liver is ca. 0.5 g lipid per hour (7). Assuming that all of this lipid was oleic acid, maximum removal would be less than 0.2 μmol/hr/g liver, which is far below the intracellular oleate activating capacity observed in this study and by other investigators (8).

The relationship between solubility and rate of activation of long chain fatty acids is unclear. Kolb and Brown concluded that solubility of the Δ^6 and Δ^9 *cis*-octadecenoates was proportional to the distance of the double bond from the carboxyl group (9). However the increased solubility that would result as the double bond is moved from carbon 9 to carbon 17 would not be correlated with the activation profile observed in the present study. In addition, Massoro and Lennarz concluded that with *B. Megaterium* acyl-CoA synthetase, fatty acid solubility was not correlated with acyl-CoA formation (10). The self-inhibition observed with the Δ^8,Δ^9 and Δ^{10} isomers may be related to the ability of their micelles to bind to the acyl-CoA synthetase(s) surface. However the relationship between positional isomerism, micelle size and shape and rate of acyl-CoA formation remains to be clarified. In addition, the exact form in which the fatty acid substrate binds to the enzyme catalytic site, i.e., fatty acid molecule, fatty acid micelle or fatty acid-carrier protein complex, remains to be elucidated. Differential rates of inhibition of activation of the isomeric octadecenoates were observed in the present study in the presence of retinoate and several detergents. These differences probably reflect the degree to which the detergent micelles incorporate the positional isomers into the micelle.

It is interesting that the Δ^7 and Δ^{13} isomers of *cis*-octadecenoic acid accumulate in position 1 of the phospholipids of fish and mammals and in positions 1 and 3 in triglycerides of mammals fed on fish. In contrast, the 9 isomers generally accumulate in position 2 (11). We

have previously found that *trans*-octadecenoates and saturated acids that also accumulate preferably in positions 1 and 3 of triglycerides have higher optimal fatty acid-protein ratios for maximum rates of activation than the corresponding *cis*-isomers (1). The optimal fatty acid acid-protein ratios for maximum activation of the positional isomers with the double bond at either end of the fatty acid molecule observed in this study were much higher than the ratio required for maximum oleate activation (Table I). Thus the isomers with carboxyl or methyl terminal double bonds apparently have different properties from the Δ^8 to Δ^{10} octadecenoates with a double bond in the middle of the fatty acid chain. Possibly the Δ^8 to Δ^{10} isomers are treated enzymatically as monounsaturated acids, while the other isomers are treated as saturated acids. This distinction in enzyme recognition would account for the difference in positional specificity observed for these isomers (11).

The *cis*-octadecenoate positional isomers did not equally affect the growth rate of monkey kidney cells grown in tissue culture (12). The Δ^3 and Δ^{17} isomers inhibited growth, while addition of the Δ^5 to Δ^{16} isomers resulted in differential stimulation of growth. Maximum stimulation of growth was noted upon addition of the Δ^5 to Δ^9 isomers. These results support the suggestion that whole cells as well as subcellular organelles differentiate between the terminal and centrally located *cis*-octadecenoate positional isomers. Differential recognition of double bond positional isomers has also been noted previously for the reactions catalyzed by acyl transferases on the isomeric octadecodienoyl CoA esters (13) and isomeric octadecenoyl CoA esters (14) and for soybean lipoxidase on polyunsaturated fatty acids (15).

Further studies are required to clarify whether the differential rates of activation and responses to various detergents presented herein reflect enzyme specificity, solution properties of the positional isomers or a combination of these factors. The parallel changes in the activation profile, with changes in pH, temperature and with aging of the enzyme, do not lend support to the suggestion that more than one acyl-CoA synthetase enzyme catalyzes acyl-CoA biosynthesis of the octadecenoate positional isomers in rat liver.

REFERENCES

1. Lippel, K., Lipids 8:111 (1973).
2. Lippel, K., F.D. Gunstone and J.A. Barve, Lipids 8:119 (1972).
3. Gunstone, F.D., and I.A. Ismail, Chem. Phys. Lipids 1:209 (1967).
4. Lowry, O.H., G.N. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193:275 (1951).
5. Bortz, W.M., and F. Lynen, Biochem. Zeit. 339:77 (1963).
6. Van Harken, D.R., C.W. Dixon and M. Heimberg, Biol. Chem. 244:2278 (1969).
7. Felts, J.M., and M.N. Berry, Biochim. Biophys. Acta 231:1 (1971).
8. Pande, S.V., and J.F. Mead, J. Biol. Chem. 243:352 (1968).
9. Kolb, D.R., and J.B. Brown, JAOCS 32:357 (1955).
10. Massoro, E.J., and W.J. Lennarz, Biochem. 4:85 (1965).
11. Brockerhoff, H., and R.G. Ackman, J. Lipid Res. 8:661 (1967).
12. Jenkin, H.M., L.E. Anderson, R.T. Holman, I.A. Ismail and F.D. Gunstone, Exp. Cell Res. 59:1 (1970).
13. Holman, R.T., P.O. Egwim and W.W. Christie, J. Biol. Chem. 244:1149 (1969).
14. Reitz, R.C., W.E.M. Lands, W.W. Christie and R.T. Holman, Ibid. 243:2241 (1968).
15. Reitz, R.C., W.E.M. Lands, M.E. Sheikh, F.D. Gunstone and I.A. Ismail, AOCS 42nd Fall Meeting, 1968, p. 48.

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Autoxidation of Polyunsaturated Fatty Esters on Silica

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ABSTRACT

The stability of unsaturated methyl esters of fatty acid adsorbed on silica gel and silicic acid was studied by gas liquid chromatographic estimation of disappearance relative to a saturated internal standard. Variables included silica-ester ratio, agitation, adsorbent particle size, and degree of unsaturation. Under the conditions of the experiment, destruction of substrate unsaturated ester at 80 C was more a function of time than of unsaturation or initial purity.

INTRODUCTION

It was recently shown (1,2) that stability of unsaturated oil and methyl esters of polyunsaturated fatty acids is greatly increased when adsorbed on silica gel. This finding was considered significant, not only from a practical viewpoint but also because of its possible implication of a mechanism of autoxidation different from that elucidated in solutions or in neat oils (3-6). It may also have a bearing on the mechanism of autoxidation of lipids in some ordered arrangement as in layers or membranes (7,8), a reaction that has been implicated in oxidative tissue damage as in the aging process (9).

In a further extension of this research, a comparison has been made of the rates of oxidation of methyl esters with different degrees of unsaturation adsorbed on silica gel. The results appear to confirm the different mechanism of autoxidation of adsorbed polyunsaturates.

MATERIALS AND METHODS

Pentane, cyclohexane, diethyl ether, silica gel (J.T. Baker 3405, lot 34110) and silicic acid (J.T. Baker 0324, lot 32080, 200-325 mesh) were reagent grade and were used without further purification. The adsorbents contained some metal impurities, as determined by emission spectroscopy. The ppm of Fe, Co, Ni, Cu, Ca and Mg were 100, not detected (ND), 0.8, 0.1, 45 and 120, respectively, for the silica gel, and 5, ND, 0.5, 0.2, 1 and 0.1, respectively, for the silicic acid. Traces of Ti, Al and Na were observed; no detectable amounts of Cr, Sr, Ba or Ag were found. Methyl linolenate, methyl eicosapentaenoate and methyl docosahexaenoate were of variable purity, which was checked, in part, by UV spectrum and gas liquid chromatographic (GLC) purity.

A rotating shaker was altered in such a way that vigorous agitation of the solid adsorbent could be achieved. The shaker was maintained at 80 C.

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TABLE I

Oxidation of Unsaturated Esters on Silica Gel at 80 C

Methyl esters	Time, days	Per cent oxidized		
		Experiment 1[a]	Experiment 2[b]	Experiment 3[b]
18:3	2	67	—	63
20:5	2	70	—	—
22:6	2	—	—	69,64
18:3	4	89	87	82
20:5	4	91	—	—
22:6	4	—	83	90

[a]Disappearance of 0.5 mg unsaturated ester on 0.5 silica gel was measured relative to 0.1 mg Me 18:0. The unsaturated esters contained significant conjugation. Gas liquid chromatography on EGS.

[b]Disappearance of 0.5-0.6 mg unsaturated esters on 0.5 g silica gel was measured relative to 0.05 mg Me 16:0 and 0.1 mg Me 20:0. Gas liquid chromatography on OV-1.

TABLE II

Effect of Gel-Unsaturated Ester Ratio

Methyl ester	Gel: Unsaturated ester, g/mg	Per cent of original oxidized[a]: 2 Days	4 Days
18:3	0.20	63	88
	1.0	67	89
	2.0	62	90
20:5	0.19	62	88
	0.92	70	91
	1.8	68	87

[a]Disappearance of 0.5 mg of unsaturated ester was measured relative to 0.1 mg Me 18:0. Gas liquid chromatography on EGS. (Experiment I oxidation values for gel-ester ratio = 1 also appear in Table I.)

Gas Liquid Chromatography

Analyses for the first experiments were performed with polar columns. A Barber-Colman Model 20 apparatus was equipped with a 6 ft x 1/8 in. 12% DEGS column. A Barber-Colman Model 10, with a flame-ionization detector was equipped with a 40 in. x 6 mm 12% EGS column. Because the retention time for 22:6 at the maximum recommended temperature (195 C) was ca. 45 min, later analyses were performed using a nonpolar column. A 40 in. x 6 mm 3% OV-1 column was used in the Barber-Colman Model 10. The retention time of Me 22:6 on the nonpolar column was 22 min at 185 C and 20 psig A.

Calculations

The progress of autoxidation was measured on GLC by the disappearance of the unsaturated methyl ester relative to that of an added saturated ester. The saturated ester was presumed unchanged, although it is recognized that saturated esters may autoxidize (10). From relative autoxidation rates of 1:100:1000 for Me 18:0, Me 18:1 and Me 18:2 (11), it was presumed that the saturated ester would be substantially unchanged as long as a trace of unsaturated ester remained.

The per cent of the original unsaturated ester remaining was estimated from the ratio unsat.-sat. after oxidation and extraction. This ratio was divided by the initial unsat.-sat. ratio and multiplied by 100. Weight ratios were used for calculation of the initial mixtures unless GLC indicated gross deviation. In these instances, GLC initial ratios were used. Single values in tables represent single chromatograms.

Procedure

The disappearance of unsaturated methyl esters relative to saturated internal standards was measured by GLC. Methyl esters were deposited on a silica support by pipetting appropriate volumes of unsaturated and saturated ester solutions into a flask containing a cyclohexane slurry of silica gel or silicic acid. Cyclohexane was used to prepare the standard solutions since they could be stored at -20 C in the solid state. It was hoped that immobilization in this manner might decrease autoxidation during storage and, indeed the poly-unsat.-sat. ratios of freshly mixed stock solutions showed no significant decrease during 6 months of storage. Concentrations of unsaturated methyl esters in the solutions were ca. 0.5 mg/ml cyclohexane.

The flasks, containing cyclohexane, silica and ester, were swirled to mix the contents and the solvent was removed under vacuum on a rotating evaporator, below 50 C. The dry powder was transferred to a clean 125 ml Erlenmeyer flask to avoid an ester film on the inside surface of the original flask.

The flasks were heated at 80 C open to air. Portions (80-200 mg) were removed at intervals with a small porcelain spoon for GLC analysis. The silica was transferred to a 9 in. glass disposable Pasteur pipette containing a small plug of pyrex wool. Esters were extracted from this small column with two or three 1 ml portions of diethyl ether. The solvent from each extraction was evaporated in a stream of nitrogen before the next extraction. The 1 ml vials containing the solvent were held in a heating block at 35 C until the solvent had just evaporated, and the resulting methyl ester mixture was subjected to GLC analysis as rapidly as possible.

RESULTS

Several types of experiments were carried out, all at 80 C. This temperature was conveniently accessible, yet high enough so that reasonably fast oxidation rates prevailed. It was first confirmed that thin films of unsaturated esters, that is, films not adsorbed on silica gel or on silicic acid but simply spread on the bottom of a 125 ml Erlenmeyer flask, underwent the normal rapid autoxidation. Samples of 0.25-0.5 mg Me 18:3 were 21% oxidized after 30 min and 63% oxidized after 1 hr at 80 C. Similar samples of Me 22:6 were 66% oxidized after 30 min and 96% oxidized after 1 hr. Esters with different degrees of unsaturation oxidize at different rates, as is usually expected. The rate is considerably more rapid than when adsorbed, since, as seen below, the same amount of either ester required 4 days for 80-90% oxidation. Thus the earlier observation of decreased surface oxidation (2) is confirmed.

Most of the experiments were carried out with the use of silica gel as the adsorbent. A first set of results is given in Table I, under run 1. We were concerned, however, that although freshly opened vials of material were used, there was evidence of contamination by oxidation products. The 18:3/18:0 ratio was 4.49 by GLC for a mixture made up to have a ratio of 4.80 w/w. Worse, the 20:5/18:0 ratio was 2.95 by GLC as compared to 5.23 w/w. Also, the UV spectra indicated the presence of some conjugated diene in the 18:3 material and of both conjugated diene and triene in the 20:5 material. It is known, of course, that in the usual autoxidation studies, impurities can have significant effects (3,6,12-14).

New samples of esters were therefore used for runs 2 and 3 of Table I. No conjugated dienes were seen in either sample. Moreover the 22:6/20:0 ratio was 5.44 by GLC and 5.52 w/w, or the same within experimental error; even better agreement was found between the 18:3/20:0 ratios. The results of the three runs are essentially the same, however, and it thus appears that the presence of oxidation impurities does not materially affect the oxidation rates of the adsorbed ester. As a precaution, however, all subsequent runs were made with the second set of samples.

A second series of experiments was planned to determine the effect of varying the ratio of ester to silica gel. The results of a 10-fold variation are summarized in Table II. No significant effect is present.

A point of interest was whether our uniformly slow oxidation rates for the adsorbed esters could possibly be a reflection of oxygen diffusion to the surface as a rate limiting step. To check this possibility, Me 18:3 and Me 22:6 adsorbed on silica gel were allowed to oxidize under conditions of vigorous shaking such that the particles were continuously tumbled. The results of the dynamic (shaken) and static (unshaken) runs are given in Table III. There appears to be no effect of greatly increasing the ease of access of oxygen to the silica gel particles.

The 2-4 day periods used for most of the runs allowed sufficient oxidation to occur for any differences in rates between the various esters to be magnified. It was desirable, however, to investigate the oxidation rate law more closely by obtaining values for the degrees of oxidation at shorter times. A set of such runs gave results summarized in Table IV. These results, being reported as per cent of original ester oxidized, necessarily exhibit more scatter than do the data for the longer times. The point is that our actual measurement is of the relative amount of ester not oxidized, and the per cent oxidation is obtained by comparing this measurement with that for an untreated or reference sample. Each measurement separately is subject to ca. 5% uncertainty in absolute value, although the precision is actually somewhat better. The effect is that the smaller the degree of reaction, the more uncertain is its actual value. A reported 10% degree of reaction is, for example, probably subject to 50% relative error. With this aspect in mind, there appears to be no significant difference in oxidation rate between the various esters, with the possible exception of the 3.5 hr results.

TABLE III

Comparison of Dynamic and Static Oxidation of Unsaturated Esters at 80 C

Methyl ester	Time, days	Per cent of original oxidized[a]	
		Dynamic	Static
18:3	2	58 (60)[b]	50 (54)[b]
22:6	2	63	63
18:3	4	88	83
22:6	4	91	88 (87)[b]

[a]Disappearance of 0.5-0.6 mg unsaturated ester on 0.5 g silica gel was measured relative to 0.05 mg Me 16:0 and 0.1 mg Me 20:0. Gas liquid chromatography on OV-1.

[b]Duplicate gas liquid chromatography value.

Finally, we wished to test for sensitivity to at least minor changes in the nature of the adsorbent. This was done by carrying out a set of runs using silicic acid. The results are given in Table V. Silicic acid appears to be a somewhat more inert supporting material than silica gel, although the effect is not large. It should be noted that the apparent ester-adsorbent ratios may not be strictly comparable between the two adsorbents. The silica gel was 60-200 mesh, while the silicic acid was 200-325 mesh; the average particle sizes and probably also the specific surface areas were thus different. In addition, the loss in weight on ignition was 8.2% for the silica gel and 23.8% for the silicic acid, according to the manufacturer's specifications. This means that the number of surface sites per gram of material was probably different for the two adsorbents, even for the same mesh size particles. Moreover the nature of such sites may have been somewhat different because of the different water content.

DISCUSSION

The above results appear to support the following immediate conclusions: (a) Oxidation rates for adsorbed unsaturated esters are one-

TABLE IV

Early Stages of Oxidation[a]

Methyl ester	Hours 80 C	Per cent oxidized	Average per cent oxidized
18:3	3.5	0, 6.8	3.4
22:6	3.5	16.6 (17.2),[b] 20.2	18.4
18:3	5.75[c]	16.5, 20.1	18.3
22:6	5.75[c]	28.8 (29.0),[b] 21.9	25.3
18:3	22[c]	48.2, 47.3	47.8
22:6	22[c]	56.5, 53.4	55.0

[a]Disappearance of 0.5-0.6 mg unsaturated ester on 0.5 g silica gel was measured relative to 0.05 mg Me 16:0 and 0.1 mg Me 20:0. Gas liquid chromatography on OV-1.

[b]Duplicate gas liquid chromatography value was not used in average to prevent weighting.

[c]Samples were removed and stored at -20 C and extracted just before analysis.

twentieth to one-thirtieth as fast as those for esters in bulk. (b) The oxidation rates of adsorbed esters are not sensitive to (c) the ester-adsorbent ratio at the level of 1 mg/g, (d) variation in the ease of access of oxygen to the surface of the particles of adsorbent, or (e) to the molecular weight or the degree of unsaturation of the ester. Finally, (f) the rate may be sensitive to the type of silicious adsorbent used.

The data were tested with various rate laws, using average values for the degrees of oxidation of each ester at each of the various times, as obtained from the individual observations reported in Tables I-IV. The results agree fairly well with the first order rate law, $A/A_o = e^{-kt}$, where A/A_o denotes the fraction of ester remaining at time t, and k is the apparent first order rate constant. The corresponding semi-logarithmic plot is shown in Figure 1. With the exception of the 3.5 hr point for Me 18:3, all of the data lie sufficiently close to a straight line for distinctions between the esters to seem unwarranted, in confirmation of point (e) above. (Somewhat better agreement does result if separate straight lines of slightly different slope are fitted to the 18:3 and the 22:6 points, but it is doubtful that the precision of the data justifies this level of detail.) The data do not fit a simple second order plot (A_o/A vs. t) at all, nor do they give a straight line when plotted as A/A_o vs. $t^{1/2}$, or as $(1-A/A_o)^2$ vs. t. These last are forms sometimes followed when diffusion through a deepening layer of material is involved (15a).

Returning to the first order plot, the line in Figure 1 is drawn so as to intercept A/A_o = 90% at t = 0; this gives a better fit to the points than does a line drawn to A/A_o = 100%. Such an intercept suggests the presence of a fast component to the rate law. This component could be bulk phase oxidation occurring during the preparation of the system, but we have no reason to believe that our procedures could have caused an acceleration of this ordinarily slow process. Another explanation is suggested below. It should be noted that the 3.5 hr point for Me 18:3 would, if given full weight, suggest a 1-2 hr induction period. A more detailed investigation would be needed to establish such a feature, however.

The results for silicic acid are included in Figure 1. If first order behavior is assumed, the oxidation rate is about two-thirds of that on silica gel. The data, while limited, are fit best by a line drawn to 100% A/A_o at t = 0, suggesting that no fast component is present in the rate law.

An aspect of possible importance to any discussion of mechanism is the following. The specific surface area of our samples is ca. 300 m^2g^{-1} (16,17). If we take the radius of 325 mesh material to be ca. $2x10^{-4}$ cm and the density of silica gel to be ca. 2.5, then for spherical particles the specific surface area is ca. 0.5 m^2g^{-1}. Thus most of the "surface" of our adsorbents must be interior surface; the materials are highly porous. The adsorbed esters are probably distributed fairly uniformly throughout the interior of each particle. A possibility that must be considered, therefore, is that a bulk-type reaction is occurring in our systems but that it is slow because of being limited in rate by that of diffusional access of the oxygen. The lack of dependence of the rate on the degree of agitation is not diagnostic, since diffusion of oxygen into and through individual particles could be the slow step. There are, however, some counterindications. Oxygen diffusion into particles should not obey a first order rate law; also qualitative observations on the temperature dependence of the oxidation indicate that it is much larger than would be expected for a gas diffusion process. For example, the earlier results (2) indicated ca. 30% oxidation of esters with three or more bonds on silica gel after 63 days at 4 C while the present results show that only ca. 1 day is required at 80 C. The comparison generates an

TABLE V

Oxidation of Unsaturated Esters on Silicic Acid at 80 C[a]

Methyl ester	Time, days	Per cent oxidized	
		Experiment 2	Experiment 3
18:3	2	—	52,45
22:6	2	—	43
18:3	4	77	75
22:6	4	73	78,76

[a]Disappearance of 0.5-0.6 mg unsaturated esters was measured relative to 0.05 mg Me 16:0 and 0.1 mg Me 20:0 on 0.5 g silicic acid. Gas liquid chromatography on OV-1.

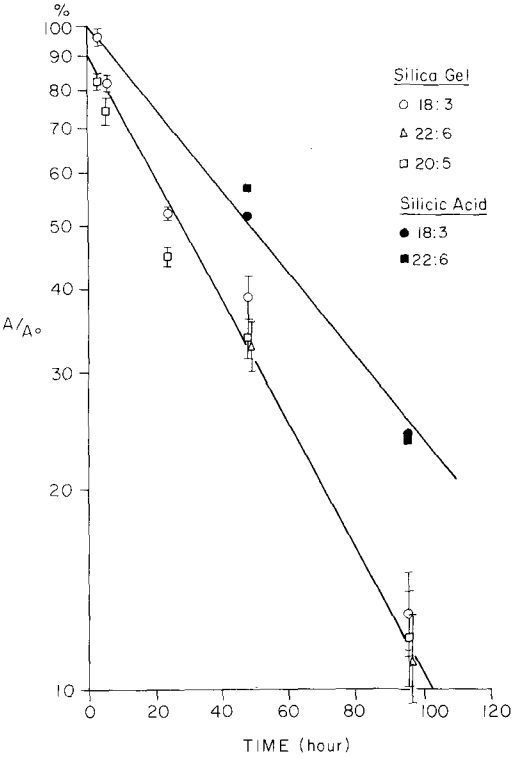

Fig. 1

apparent activation energy of 11 kcal mol^{-1}. Gas diffusion coefficients are proportional to the average molecular velocity and hence to $T^{1/2}$ (18). Another point is that the more unsaturated esters should provide a deeper oxygen "sink"; more oxygen would have to diffuse into a particle for a given degree of loss of original ester and a slower rate of disappearance would be expected, contrary to our observation. Finally, the absolute rates are rather small to be explained in terms of gas diffusion. The reaction itself does not appear to be of the bulk type, since we observe little if any induction period.

Our tentative conclusion is that the rate limiting step is a direct chemical reaction between oxygen and adsorbed ester, uncomplicated by the free radical chains involved in the bulk reaction (4). A calculation is instructive at this point. A loading of 1 mg ester per gram of adsorbent of surface area 300 m^{2}g^{-1} corresponds to an apparent film thickness of ca. 0.03 Å. The actual thickness of a hydrocarbon chain is ca. 3 Å so that only ca. 1% of the surface is actually occupied. Adsorbed ester molecules are thus far apart on the average and it is very unlikely that they would be able to participate in a cooperative or chain process. A first order rate law should be obeyed since oxygen diffusion is now not considered to be rate controlling. There should be no induction period.

We next consider the possible nature of the oxidation step itself. One possibility is that the adsorbent inhibits oxidation by isolating the ester molecules from each other so that no chain reaction is possible. The observed rate might then be similar to that of the *initiation* step of the bulk reaction. The difficulty with this picture is that the rate of oxidation should be at least approximately proportional to the number of double bonds in the ester molecule, contrary to observation.

The above difficulty is resolved if active sites are present, capable of catalyzing oxidation at a double bond, with only one double bond activated per molecule of ester. The rate of oxidation would now conform to observation (3). The restriction to one activated double bond per molecule can be accounted for in at least two ways. One possibility is that the spacing between activating adsorption sites does not match the distance between double bonds, so that only one bond in a given molecule can be optimally adsorbed.

An alternative possibility is that the active sites are those of metal impurities. Metal oxides are catalysts for the oxidation of various organic molecules, and of course metal ions are known to be catalysts in the homogeneous autoxidation of unsaturated esters (14,20). Although the free radical aspect of autoxidation is usually emphasized, the possibility of nonfree radical initiation in the presence of trace metal impurities has been considered (21,22). Transition metals such as cobalt, manganese, nickel, iron and copper are especially active. This possibility could take one of two forms. Ester adsorption might be localized on metal oxide sites, but with the sites far enough apart that only one double bond per molecule is activated. This would be a stoichiometric

mechanism. Less than a stoichiometric number of sites would be needed, however, if surface diffusion allowed ester molecules to migrate to a catalytic site for reaction. For first order kinetics to be observed, it would be necessary for the site reaction to be the slow step. Not much is known about surface diffusion rates on solids, but indications are that they can be quite appreciable (15b), so this dynamic mechanism may not be unreasonable.

The 100 ppm of iron in the silica gel corresponds, on a mole basis, to ca. 0.5 mg of unsaturated ester. If the iron were entirely on the surface, this amount would barely suffice to provide the adsorption sites necessary for the stoichiometric mechanism; the amount would probably be sufficient to account for the possible fast component in the oxidation, however. Neither iron nor the other likely candidates, nickel and cobalt, are present in sufficient amount in either adsorbent to hold the quantities of ester used. While the stoichiometric mechanism thus seems to be ruled out, the dynamic one could still apply. It is of course suggestive that the silicic acid was less active than the silica gel and also contained smaller amounts of transition metal impurities.

We believe that the present results establish a potentially important phenomenon, namely the reduced rate and nonautocatalytic nature of the oxidation of adsorbed unsaturated esters. A number of additional parameters remain to be studied. These include the dependence of the oxidation rate on the partial pressure of the oxygen, on temperature, on the degree of hydration (or of heat treatment) of the adsorbent, and on the surface concentration of metal impurities. Our present data may reflect this last parameter: The disappearance of the fast component to the rate law on going from silica gel to silicic acid could be due to there being a smaller concentration of some active impurity in the latter adsorbent.

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REFERENCES

1. Honn, F.J., I.I. Bezman and B.F. Daubert, JAOCS 28:129 (1951).
2. Slawson, V., and J.F. Mead, J. Lipid Res. 13:143 (1972).
3. Farmer, E.H., H.P. Koch and D.A. Sutton, J. Chem. Soc. (London) 1943:591.
4. Bolland, J.L., Quart. Rev. Soc. (London) 3:1 (1949).
5. Bateman, L., Quart. Rev. Chem. Soc. (London) 18:147 (1954).
6. Bateman, L., H. Hughes and A.L. Morris, Dis. Faraday Soc. 14:190 (1953).
7. Togashi, H.J., A.S. Henick and R.B. Koch, J. Food Sci. 26:186 (1961).
8. Porter, W.L., L.A. Levasseur and A.S. Henick, Lipids 7:699 (1972).
9. Pryor, W.A., Sci. Amer. 223:70 (1970).
10. Brodnitz, M.H., W.W. Nawar and I.S. Fagerson, Lipids 3:59 (1968).
11. Silbert, L.S., JAOCS 39:480 (1962).
12. Privett, O.S., and M.L. Blank, Ibid. 39:465 (1962).
13. Vioque, A., R. Gutierrez, M.A. Albi and N. Nosti, Ibid. 42:344 (1965).
14. Uri, N., Nature 177:1177 (1956).
15. Adamson, A.W., "Physical Chemistry of Surfaces," Second edition, Interscience Publishers, New York, London, 1967, (a) p. 309, (b) p. 197, 265.
16. Snyder, L.R., "Principles of Adsorption Chromatography," Dekker, New York, 1968, p. 163.
17. Gregg, S.J., and K.S.W. Sing, "Adsorption, Surface Area, and Porosity," Academic Press, New York, 1967, p. 82.
18. Hirschfelder, J.O., C. Curtiss and R.B. Bird, "Molecular Theory of Gases and Liquids," Corrected edition, John Wiley and Sons, Inc., New York, 1964, p. 527.
19. "Fundamentals of Gas-Surface Interactions," Edited by H. Saltsburg, J.N. Smith and M. Rogers, Academic Press, New York, 1967, p. 239.
20. Ingold, K.U., "Metal-Catalyzed Lipid Oxidation," SIK-Report 240, Edited by R. Marcuse, Kallebäck, Goteborg, Sweden, 1968.
21. Uri, N., "Autoxidation and Antioxidants," Vol. 1, Edited by W.O. Lundberg, Interscience Publishers, New York, 1961, p. 101.
22. Heaton, F.W., and N. Uri, J. Lipid Res. 2:152 (1961).

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Quantification of Lowered Cholesterol Oxidation in Guinea Pigs with Latent Vitamin C Deficiency

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ABSTRACT

Fifty-two male guinea pigs fed on a scorbutigenic diet were divided into a control group (10 mg ascorbic acid per animal per day) and a group with latent vitamin C deficiency (2 weeks on the scorbutigenic diet only, followed by a maintaining dose of 0.5 mg ascorbic acid per animal per day). After 13 weeks, 26-^{14}C-cholesterol was administered intraperitoneally to all the animals, in which the ^{14}C excretion in the expired CO_2 and the urine and cholesterol specific activity in the blood serum and liver were then studied at intervals of 24 hr and 1, 3, 5, 7, 9 and 11 weeks. The ascorbic acid concentration in the liver and spleen of the control animals was five times higher than in vitamin C-deficient animals. The total cholesterol concentration in serum and liver was significantly higher in the vitamin C-deficient guinea pigs. A two-pool analysis of the disappearance curves of serum cholesterol specific activity showed that the size of the cholesterol pool A (blood and tissues with rapid cholesterol exchange) was greater in the vitamin C-deficient animals. The rate of the transformation of cholesterol to bile acids was estimated as the ratio of $^{14}CO_2$ expired to liver cholesterol specific activity. Latent vitamin C deficiency caused significant slowing down of this process (controls: 11.8 ± 0.6; vitamin C deficiency: 8.3 ± 0.4 mg/24 hr/500 g w/w). A significant correlation between the liver ascorbic acid concentration and the rate of cholesterol transformation to bile acids was found. The results demonstrate that ascorbic acid is necessary for a normal course of cholesterol catabolism. In latent vitamin C deficiency, the rate of cholesterol catabolism slows down and cholesterol consequently accumulates in the blood and liver of vitamin C-deficient guinea pigs.

INTRODUCTION

In guinea pigs with chronic latent ascorbic acid deficiency, we observed reduced incorporation of label from 4-^{14}C-cholesterol into bile acids and lower oxidation of 26-^{14}C-cholesterol to $^{14}CO_2$ (1). Since the results of these experiments were expressed in terms of the percentage of administered radioactivity, without considering serum or tissue cholesterol specific activity, the data might have been influenced by differences in the size of the cholesterol pools in the control and vitamin C-deficient animals. The aim of the present study was to determine the transformation of 26-^{14}C-cholesterol to bile acids in normal and vitamin C-deficient guinea pigs quantitatively by studying $^{14}CO_2$ output and cholesterol specific activity by a method which proved satisfactory in man (2).

EXPERIMENTAL PROCEDURES

Fifty-two male guinea pigs with an initial weight of ca. 330 g, fed ad libitum on a scorbutigenic diet (3), were divided into a control and a vitamin C-deficient group. The controls received 10 mg ascorbic acid per animal per day perorally by tube, while hypovitaminosis C was induced by 14 days' administration of the scorbutigenic diet without added vitamin C, followed by the peroral administration of a maintaining dose of 0.5 mg ascorbic acid per animal per day (4). After 13 weeks, when the animals weighed ca. 500 g and their weight curve had started to flatten out, 26-^{14}C-cholesterol (The Radiochemical Centre, Amersham, U.K.; specific acitivity 55 mCi/mM), emulsified with Tween 20 in sterile saline, was administered intraperitoneally to each of the animals in a dose of 1.2 μCi/100 g body wt. The animals were subsequently fed on the same diet as before. Immediately after injecting labeled cholesterol, three animals from each group were placed in all-glass metabolic cages with forced ventilation, which allowed quantitative collection of the feces, urine and expired CO_2 (5). The guinea pigs had access to food and water while in the cage. After 24 hr the two groups were killed by decapitation, the blood serum extracted with ethanol-ether 3:1 and the total cholesterol concentration determined by the Liebermann-Burchard reaction (6). Total ^{14}C activity was determined in another serum aliquot and in the urine, after hydrolysis in Nuclear Chicago Solubilizer. The liver and various tissues were extracted with

TABLE I

Total Cholesterol Concentrations in Control Guinea Pigs and Guinea Pigs with Chronic Latent Hypovitaminosis C

Tissue	mg/100 g Wet tissue or 100 ml serum		Statistical significance
	Control	Hypovitaminosis C	
Liver	359 ± 15 (26)[a]	443 ± 19 (26)	$P < 0.002$
Kidney	325 ± 6 (26)	325 ± 6 (25)	NS[b]
Adrenal	7269 ± 384 (26)	6844 ± 461 (26)	NS
Small intestine	238 ± 7 (26)	231 ± 5 (26)	NS
Large intestine	265 ± 6 (26)	268 ± 7 (25)	NS
Stomach	329 ± 7 (26)	331 ± 6 (25)	NS
Lung	401 ± 8 (26)	412 ± 6 (25)	NS
Heart muscle	163 ± 4 (26)	169 ± 3 (25)	NS
Skeletal muscle	86 ± 3 (26)	91 ± 3 (25)	NS
Brain	1659 ± 32 (26)	1658 ± 37 (25)	NS
Testis	228 ± 7 (26)	233 ± 7 (25)	NS
Epididymal fat	111 ± 5 (26)	122 ± 35 (25)	NS
Skin	171 ± 6 (26)	211 ± 8 (26)	$P < 0.001$
Thoracic aorta	392 ± 46 (26)	405 ± 42 (26)	NS
Blood serum	126 ± 9 (26)	218 ± 17 (26)	$P < 0.001$

[a]Numbers in parenthesis denote the number of animals observed.
[b]NS = not significant ($P > 0.05$).

chloroform-methanol 2:1, and the total cholesterol concentration and total ^{14}C activity in the extracts determined. Since we found (7,28) that the total activity of liver lipid extracts from guinea pigs injected with 26-^{14}C-cholesterol was identical with that of isolated ^{14}C-digitonides, we used these data for calculating cholesterol specific activity. The ^{14}C content of the expired CO_2 was determined in an apparatus after Saba and Di Luzio (8) by our own modified technique (5). Radioactivity was measured in a scintillation spectrometer (Mark I, Nuclear Chicago) at 0 C with an external standard for quenching correction. The vitamin C concentration was determined in the liver and the spleen (9). The same data were obtained successively from groups of three to four control and three to four vitamin C-deficient guinea pigs killed at intervals of 1, 3, 5, 7, 9 and 11 weeks after injecting labeled cholesterol.

The rate of cholesterol transformation to bile acids was estimated as the ratio of the 24 hr disintegration count in the expired $^{14}CO_2$ to the simultaneously determined cholesterol specific activity in the liver, i.e., in the organ in which this process takes place. The die-away curve of cholesterol specific activity in the serum was subjected to a kinetic two-pool analysis (10). The results were evaluated statistically by Student's t test and linear correlations by the method of the least squares. The values of the partial and multiple correlation coefficients were also determined. Unless explicitly stated otherwise, the results are given in the tables as the means ± the standard error of the mean.

RESULTS

In the preparatory phase before administering labeled cholesterol, the body weight of both the control and the vitamin C-deficient guinea pigs rose. The weight increments in the two groups were similar (control + 180 g, vitamin C-deficient + 140 g; the difference is not statistically significant). In the interval between

TABLE II

Vitamin C Concentration in Liver and Cholesterol Transformation to Bile Acids in Normal and Hypovitaminous Guinea Pigs Killed at Various Intervals After Administration of 26-^{14}C-Cholesterol

Weeks after administration of labeled cholesterol	Vitamin C in liver, mg/100 g wet tissue		Cholesterol transformation to bile acids, mg/animal/day	
	Control	Hypovitaminosis C	Control	Hypovitaminosis C
1	8.4 ± 1.0 (3)	1.3 ± 0.4[a] (3)	13.9 ± 1.2 (3)	7.1 ± 0.8[a] (3)
3	7.0 ± 1.1 (4)	2.0 ± 0.1[a] (3)	11.2 ± 1.7 (4)	7.1 ± 0.1 (3)
5	8.6 ± 1.6 (4)	1.6 ± 0.2[a] (3)	10.7 ± 2.4 (4)	9.0 ± 0.4 (3)
7	10.2 ± 0.8 (4)	1.7 ± 0.1[a] (3)	16.0 ± 1.3 (4)	9.4 ± 1.7[a] (3)
9	8.2 ± 0.9 (4)	2.0 ± 0.2[a] (4)	15.5 ± 1.2 (4)	9.1 ± 1.5[a] (4)
11	7.5 ± 0.6 (4)	1.1 ± 0.1[a] (5)	14.1 ± 1.0 (4)	9.8 ± 1.5[a] (5)
Mean ± SD[c]	8.3 ± 1.9 (23)	1.6 ± 0.5 (21)	13.6 ± 3.4 (23)	8.7 ± 2.8 (21)
Statistical evaluation	$t = 15.589$ $P < 0.001$		$t = 5.266$ $P < 0.001$	

[a]Significantly different from the control group ($P < 0.05$).
[b]The numbers in parenthesis denote the number of animals observed.
[c]SD = standard deviation.

injecting labeled cholesterol and killing the animals, the weight of the two groups slowly continued rising. Food consumption by the vitamin-deficient animals and their outward appearance and behavior were completely normal.

Since the total cholesterol concentrations in guinea pigs killed at different intervals after the injection of labeled cholesterol did not differ significantly, the results are given together in Table I. Hypovitaminosis C caused a highly significant increase in the serum and liver total cholesterol concentration. The level of Liebermann-Burchard positive substances likewise rose moderately in the skin of vitamin-deficient animals, but because of the incidence of various sterols in this tissue, we cannot say whether cholesterol was responsible for the increment. In all the other tissues analyzed, the total cholesterol levels in the two groups were practically the same.

The mean vitamin C concentration in the liver and spleen of the controls was five times higher than in the vitamin-deficient group (liver: control 8.2 ± 0.4, deficiency 1.6 ± 0.1; spleen: control 21.6 ± 0.8, deficiency 4.7 ± 0.2 mg/100 g fresh tissue; $P < 0.001$ in both cases). The partial mean vitamin C levels in the liver of hypovitaminous guinea pigs killed at the different intervals were very similar (Table II), showing that the given nutritional regimen formed a clearly defined state in the experimental animals.

Figure 1 illustrates the course of cholesterol specific activity in the liver and ^{14}C excretion in the CO_2 expired and in the urine by the controls. The curves for the vitamin C-deficient group have a similar course, but they show a slight shift towards lower ^{14}C loss in the CO_2 and urine and higher liver cholesterol specific activity. The curves had a parallel course from the first week after administering labeled cholesterol, with the result that the $^{14}CO_2$ (dpm/24 hr) to liver cholesterol specific activity (dpm/mg) ratio varied by less than ± one standard deviation throughout the whole of the experiment (Table II). Since the value of this ratio expresses the rate of the transformation of cholesterol to bile acids (mg/24 hr), the given results indicate that the rate of this process did not alter significantly from the first week of the experiment right to the end. The data for cholesterol catabolism obtained 24 hr after injecting labeled cholesterol are unreliable because of rapid decline of serum and liver cholesterol specific activity, and they were not included in the total series.

Table II demonstrates that both the liver vitamin C concentration and the rate of cholesterol transformation to bile acids was significantly lower, at almost all the given intervals, in the vitamin C-deficient guinea pigs than in the

TABLE III

Kinetic Analysis of Serum Die-Away Curves in Normal and Hypovitaminous Guinea Pigs Following Injection of 26-^{14}C-Cholesterol

Parameter	Control	Hypovitaminosis C
M_A, mg (size of pool A)[a]	662	726
PR_A, mg/day (production rate in pool A)[b]	19.8	19.5
k_{AA}, day^{-1} (rate constant for the total removal of cholesterol from pool A)	-0.0400	-0.0325
k_A, day^{-1} (rate constant for the irreversible removal of cholesterol from pool A)	0.0299	0.0268
k_{AB}, day^{-1} (rate constant for the transport of cholesterol from pool A to pool B)	0.0101	0.0057

[a]Cholesterol in blood and tissues which are rapidly equilibrated with blood.
[b]Rate of entry of new cholesterol to pool A.

relevant controls. The mean rate of cholesterol transformation to bile acids for the whole experiment was very significantly lower in the vitamin-deficient group than in the controls (bottom part of Table II). Since the mean body weight of the controls was somewhat higher than that of the vitamin-deficient groups, we related the resultant values for each animal to a uniform body weight of 500 g. Even so, we found that the control guinea pigs catabolized ca. 40% more cholesterol daily than animals with chronic latent vitamin C deficiency (controls 11.8 ± 0.6, vitamin C-deficient animals 8.3 ± 0.4 mg/24 hr/500 g w/w; $P < 0.001$).

Correlation analysis showed that the rate of cholesterol catabolism to bile acids in guinea pigs was in inverse proportion to the serum cholesterol level ($r_{yx_1} = -0.524$, $P < 0.001$). Since cholesterol is transformed to bile acids in the liver, we correlated the rate of this process with ascorbic acid concentration in this organ. We found that an increase in the liver ascorbic acid concentration was accompanied by a linear increase in the rate of the transformation of cholesterol to bile acids ($r_{x_1x_2} = + 0.580$, $P < 0.001$) (Fig. 2). If we took the simultaneous action of these two factors (the cholesterol transformation to bile acids and the liver vitamin C concentration) on the serum cholesterol level into account, the multiple correlation coefficient was found to be even higher ($R_{y.x_1x_2} = 0.588$, $P < 0.001$).

The disappearance curves of blood serum specific activity in guinea pigs could be described in terms of a two-pool model similar to the one described by Goodman and Noble (10) for the plasma cholesterol turnover in man. We therefore applied the kinetic analysis for two-pool systems to our data. The results are presented in Table III. The terms used here have been defined previously (10,11). The results indicate that the rate of entry of cholesterol into the pool with rapid metabolism (PR_A) is practically the same in the vitamin C-deficient groups as in the control, whereas the values of the rate constants for the removal of cholesterol from pool A (rapid exchange of cholesterol with plasma) to pool B (slow exchange of cholesterol with plasma) and its removal from the system (k_{AB} and k_A) are lower in vitamin C-deficient guinea pigs. The size of pool A (M_A) is greater in the vitamin-deficient group, despite the fact that the mean body weight of the vitamin C-deficient guinea pigs was lower than that of the controls. Since statistical evaluation of these data was impossible (the individual points of the die-away curves were obtained from different animals killed at different intervals), we regard the results of the kinetic two-pool analysis as merely preliminary.

DISCUSSION

The results of our previous studies (1,4) drew attention to a new metabolic function of vitamin C, i.e., that ascorbic acid is required for normal transformation of cholesterol to its main catabolic product—bile acids. The results of the present study demonstrate conclusively that the rate of cholesterol transformation to bile acids slows down in the presence of chronic latent vitamin C deficiency. Adult male guinea pigs weighing ca. 500 g catabolize 3-4 mg less cholesterol daily, as a result of latent vitamin C deficiency, than control animals of the same weight with an adequate ascorbic acid intake. The vitamin C-deficient organism is evidently unable to compensate completely for this metabolic disturbance by some other homeostatic mechanism (in vitamin C-deficient guinea pigs only the absorption of exogenous cholesterol

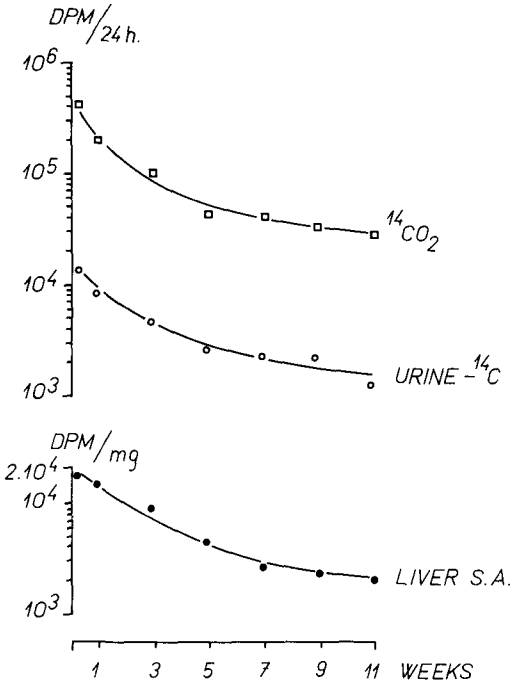

FIG. 1. ^{14}C excretion in expired CO_2 and urine and cholesterol specific activity in liver of normal guinea pigs injected with a single dose of 26-^{14}C-cholesterol.

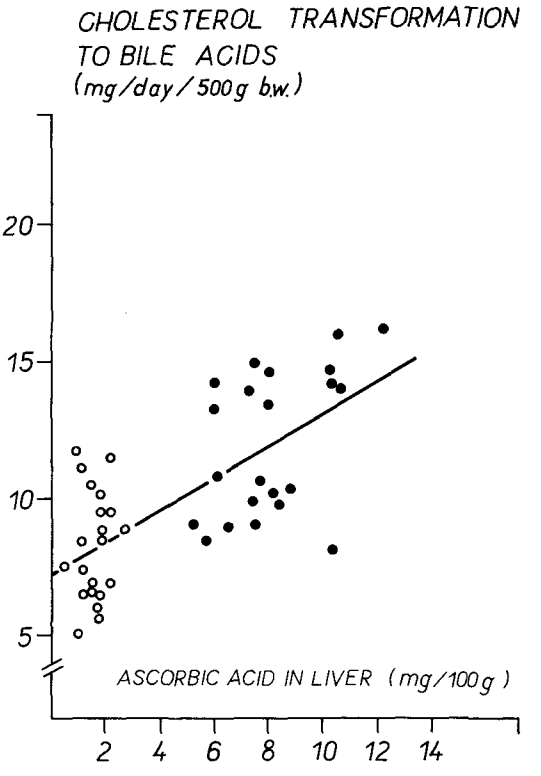

FIG. 2. Linear correlation between liver ascorbic acid concentration and the rate of cholesterol transformation to bile acids in control guinea pigs (•) and guinea pigs with latent hypovitaminosis C (○). Statistical significance: $P < 0.001$.

falls) (12), so that cholesterol accumulates in the serum and liver. The results of the two-pool analysis of the serum ^{14}C-cholesterol die-away curves also show an increase in the size of pool A, which includes serum and liver cholesterol, in the vitamin C-deficient group.

It should be emphasized that these results were obtained in guinea pigs with latent hypovitaminosis C and not in animals with acute avitaminosis C. Acutely scorbuitic guinea pigs are characterized by severe disorganization of metabolism, of which vitamin C deficiency is the primary cause, but to which a whole series of nonspecific factors, such as refusal of food, an abrupt drop in body weight, a negative nitrogen balance, morphological changes in the viscera, hemorrhage-induced anemia, etc., contribute secondarily. The pair-feeding technique only partly simulates this complex pathological state, so that the results of authors who used scurvy as model of vitamin C deficiency and found a whole series of disturbances of cholesterol metabolism (13-18) are difficult to interpret. For instance, several authors found raised cholesterol concentrations in some of the organs and in the whole body of animals with acute scurvy (13-17). This phenomenon is caused by increased cholesterologenesis, demonstrated in guinea pigs with acute avitaminosis C by raised incorporation of 1-^{14}C-acetate into liver and adrenal cholesterol (19). The mechanism of this metabolic disorder is probably as follows: Total disorganization of metabolism in acute scurvy is also associated with dysfunction of the tricarboxylic acid cycle (20,21). Since a large portion of the acetate pool is metabolized by this route, dysfunction of the tricarboxylic acid cycle causes more acetate to be metabolized by another route, i.e., by raised cholesterologenesis, in the scorbutic organism (4,19). The metabolic situation in latent ascorbic acid deficiency is quite different. The oxidation of 1-^{14}C-acetate to $^{14}CO_2$ follows a completely normal course in hypovitaminous guinea pigs, and cholesterol synthesis in their liver is likewise similar to synthesis in normal animals (22). The raised serum and liver cholesterol concentrations found in hypovitaminous guinea pigs are therefore not the outcome of altered metabolism of acetate pool. The advantage of our model of chronic latent ascrobic acid deficiency is that it does not involve total disorganization of metabolism. When interpret-

ing the results, the only variable we need to take into account is the significantly depressed ascorbic acid level in the blood and tissues of the vitamin-deficient animals. Furthermore this situation is significantly closer to the human nutritional situation in civilized communities.

Although the results convincingly demonstrated lower cholesterol transformation to bile acids in vitamin C-deficient guinea pigs, the values found for the rate of this process are somewhat lower than in actual fact. Estimation of the rate of cholesterol breakdown in man and the rat by the measurement of $^{14}CO_2$ output after administration of 26-^{14}C-cholesterol gives somewhat lower results than the use of 4-^{14}C-cholesterol (2,23). The liver of rats given 26-^{14}C-cholesterol eliminates through the bile labeled acidic compounds which are also found in the excrements (23). When bile acids are formed by degradation of the cholesterol side chain, the three end carbon atoms are removed as propionyl-CoA (24), which is rapidly oxidized to CO_2. However not all the ^{14}C split off during the transformation of 26-^{14}C-cholesterol to bile acids appeared in the expired CO_2. Malinow et al. (25) assumed that an important fraction of the side chain of cholesterol would be converted to CO_2 only after previous conversion to glucose. It is known that propionate is readily converted to liver glycogen (26) and to long chain fatty acids (27). In guinea pigs injected with 26-^{14}C-cholesterol, we found measurable ^{14}C activity in the liver glycogen and relatively high activity, probably belonging to ^{14}C-fatty acids, in the fraction of epididymal fat extract not precipitable by digitonin (28). A small proportion of the CO_2 produced by oxidation of propionate (2-3%) must be used for the synthesis of urea (2). This small percentage may account for most of the radioactivity recovered in the urine of guinea pigs injected with 26-^{14}C-cholesterol (Fig. 1). Since we found a highly significant correlation between urinary ^{14}C excretion and $^{14}CO_2$ output ($r_{xy} = + 0.812$, $P < 0.001$), it is possible that the ^{14}C excreted in the urine comes from the same pool as expired $^{14}CO_2$. The amount of ^{14}C excreted in the urine, like the amount of cholesterol catabolized via CO_2, was significantly smaller in vitamin C-deficient animals ($P < 0.01$). Since we did not attempt to determine which of the compounds in the urine contained ^{14}C, detailed interpretation of this observation is at present still impossible.

In general, however, underestimation of cholesterol transformation to bile acids, which is related to the complex metabolic fate of ^{14}C-propionyl-CoA, is not very great and does not exceed 14% of the actual rate of cholesterol conversion to bile acids (23). Despite this deficiency of the method we employed, we are of the opinion that the results convincingly demonstrate that the rate of cholesterol transformation to bile acids is directly dependent on the liver ascorbic acid concentration. Further evidence in support of the claim that a slow rate of this process is a specific consequence of vitamin C deficiency is provided by the results of a study, in which we investigated the oxidation of cholesterol in vitamin C-deficient guinea pigs resaturated with large doses of ascorbic acid. We found that resaturation with ascorbic acid significantly raised the rate of oxidation of 26-^{14}C-cholesterol to $^{14}CO_2$ (29).

Since the transformation of cholesterol to bile acids is a multistage process—which takes place in the microsomes, supernatant fraction and mitochondria of the liver cell, successively, and comprises hydroxylation, dehydrogenation, saturation of the nuclear double bond, reduction of 3-ketone and ω- and β- oxidation of the side chain (30,31)—it is difficult to decide by what mechanism ascorbic acid participates in the catabolism of cholesterol. By analogy with the general function of ascorbic acid in hydroxylation reactions, we suggested, as a working hypothesis, that ascorbic acid is necessary for the hydroxylation of cholesterol (1). The preliminary results indicate that the effect of ascorbic acid on cholesterol catabolism may be mediated by its action on the cytochrome P-450 level in the liver cell microsomes (32,33).

REFERENCES

1. Ginter, E., J. Červeň, R. Nemec and L. Mikuš, Amer. J. Clin. Nutr. 24:1238 (1971).
2. Myant, N.B., and B. Lewis, Clin. Sci. 30:117 (1966).
3. Ginter, E., R. Ondreička, P. Bobek and V. Šimko, J. Nutr. 99:261 (1969).
4. Ginter, E., "The Role of Ascorbic Acid in Cholesterol Metabolism," Slovak Acad. Sci., Bratislava, 1970.
5. Nemec, R., J. Červeň and E. Ginter, Physiol. Bohemoslov. 20:281 (1971).
6. Hořejší, J., M. Kandráč, Č. Michalec and K. Slavík, "Základy chemického vyšetrování v lékařství" SZN, Prague, 1957.
7. Bobek, P., E. Ginter and J. Červeň, Physiol. Bohemoslov. 20:523 (1971).
8. Saba, T.M., and N.R. Di Luzio, J. Lipid Res. 7:566 (1966).
9. Roe, J.H., and C.A. Kuether, J. Biol. Chem. 147:399 (1943).
10. Goodman, D.S., and R.P. Noble, J. Clin. Invest. 47:231 (1968).
11. Nestel, P.J., H.M. Whyte and D.S. Goodman, Ibid. 48:982 (1969).
12. Ginter, E., J. Červeň and L. Mikuš, Physiol. Bohemoslov. 18:459 (1969).
13. Lahiri, S., and S. Banerjee, Proc. Soc. Exp. Biol. (N.Y.) 91:583 (1956).

14. Banerjee, S., and H.D. Singh, J. Biol. Chem. 233:336 (1958).
15. Banerjee, S., and P.K. Ghosh, Amer. J. Physiol. 199:1064 (1960).
16. Kawishwar, W.K., B. Chakrapani and S. Banerjee, Ind. J. Med. Res. 51:488 (1963).
17. Phillips, W.E.J., Can. J. Biochem. 45:749 (1967).
18. Guchhait, R., B.C. Guha and N.C. Ganguli, Biochem. J. 86:193 (1963).
19. Becker, R.R., H.B. Burch, L.L. Salomon, T.A. Venkitasubramanian and C.G. King, J. Amer. Chem. Soc. 75:2020 (1953).
20. Takeda, Y., and M. Hara, J. Biol. Chem. 214:657 (1955).
21. Banerjee, S., and W.K. Kawishwar, Ibid. 234:1347 (1959).
22. Ginter, E., and R. Nemec, J. Atheroscler. Res. 10:273 (1969).
23. Chevallier, F., and C. Lutton, Bull. Soc. Chim. Biol. 48:295 (1966).
24. Suld, H.M., E. Staple and S. Gurin, J. Biol. Chem. 237:338 (1962).
25. Malinow, M.R., N. Baker, P. McLaughlin and A. Perley, J. Lipid Res. 12:747 (1971).
26. Lorber, V., W. Sakami and H.G. Wood, J. Biol. Chem. 183:531 (1950).
27. James, A.T., G. Peeters and M. Lauryssens, Biochem. J. 64:726 (1956).
28. Ginter, E., J. Jankela, R. Nemec and P. Bobek, Csl. Gastroenterol. 26:304 (1972).
29. Ginter, E., R. Nemec and P. Bobek, Brit. J. Nutr. 28:205 (1972).
30. Elliott, W.H., and P.M. Hyde, Amer. J. Med. 51:568 (1971).
31. Boyd, G.S., and I.W. Percy-Robb, Ibid. 51:580 (1971).
32. Leber, H.W., E. Degkwitz and H. Staudinger, H.S. Z. Physiol. Chem. 351:995 (1970).
33. Ginter, E., and R. Nemec, Physiol. Bohemoslov. 21:539 (1972).

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Free Fatty Acid Metabolism during Stress: Exercise, Acute Cold Exposure, and Anaphylactic Shock

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ABSTRACT

Rates of turnover and oxidation of plasma free fatty acid (FFA) were determined in unanesthetized dogs during exercise, acute cold exposure and anaphylactic shock, with the aid of a technique involving the continuous infusion of albumin-bound palmitate-1-^{14}C and the simultaneous measurement of O_2 uptake, CO_2 output and the specific activities of CO_2 and FFA. During exercise in normal untrained dogs, plasma FFA supplied 20-30% of the energy, whereas in trained dogs 70-90% of the energy was derived from the FFA oxidation. In resting dogs at room temperature the plasma FFA level was 0.58 μEq/ml with a turnover rate of 18.6 μEq/kg/min of which 22% was immediately oxidized and contributed 29% to the exhaled CO_2. These results were compared with data obtained in pancreatectomized and thyroidectomized dogs. Acute cold exposure (temperature 4-5 C) increased the FFA level and turnover rate to 1.02 μEq/ml and 28.0 μEq/kg/min, respectively, of which 33% was immediately oxidized, contributing 46% to the exhaled CO_2. During anaphylactic shock, blood lactate increased, FFA level and turnover rate were reduced, and the fraction which was immediately oxidized was depressed markedly, i.e., 3-9% of FFA turnover. Sodium lactate infusion, which induces a blood lactate level comparable to that seen in anaphylaxis or nicotinic acid infusion, markedly decreased the level and turnover rate of FFA. However the fraction of turnover oxidized remained the same as during the preinfusion period (range of 21-40%. Exercise or the administration of norepinephrine during anaphylactic shock provided results suggesting that endogenous lactic acid interferes with FFA oxidation, whereas exogenous lactate had no effect on this oxidation.

INTRODUCTION

With the discovery that tissues, particularly muscle, can take up and oxidize free fatty acids, and in spite of its small pool size, free fatty acid (FFA) has a rapid turnover rate, it became evident that both plasma glucose and FFA play an important role in energy metabolism. The two major energy depots are fat and glycogen. Fat, in the form of triglycerides, is stored primarily in adipose tissue, in the periphery, in the splanchnic area and, to some extent, in and around muscle. Glucose is stored in limited quantity as glycogen in liver and muscle. Glucose and FFA are mobilized from these energy depots and delivered to their sites of utilization via the circulatory system. Armstrong et al. (1) and Issekutz et al. (2) showed that changes in plasma FFA level were caused by alteration in the mobilization of FFA during rest and exercise and that the rate of removal was the result of a mass action effect of the plasma FFA concentration. More recently, it was shown by Paul (3,4) that animals exercising at different work loads but at equivalent plasma FFA concentrations have an increase rate of FFA turnover and percentage of FFA uptake immediately oxidized at the higher work loads. Therefore it is evident that the metabolic rate is also one of the factors to be considered when evaluating FFA metabolism. The central nervous system and hormones are essential elements of the regulatory mechanisms involved in the coordination of metabolic processes. It has been firmly established that plasma FFA is the most important energy yielding substrate for most animal tissues and that its mobilization is under sensitive hormonal control. In this paper we discuss FFA metabolism under different metabolic conditions with emphasis on the physiologic stress of exercise or acute cold exposure and on the pathologic stress of anaphylactic shock. The results will be compared with data obtained in normal animals surgically deprived of their pancreas or thyroid or infused with lactic acid, norepinephrine or nicotinic acid, agents known to affect lipid metabolism. The infusion studies were performed in order to investigate the role of altered circulating substrate levels on FFA metabolism.

EXPERIMENTAL PROCEDURES

Exercise

Normal dogs of both sexes between 10 and 20 kg were used in these studies. Untrained dogs were used for short periods of exercise lasting for 30-40 min. For longer experiments,

dogs selected for their ability to run were trained to exercise on a treadmill for 4 hr. Experiments on trained and untrained animals consisted of a 110 min rest period during which expired air and arterial blood samples were collected at 60, 75, 90 and 105 min, followed by exercise at a slope of 15% and a speed of 100 m/min during which samples were collected every 10 min in untrained and every 30 min in trained dogs.

Acute Cold Exposure

Acute cold exposure experiments were conducted on normal dogs in a ventilated climatic chamber. The control period consisted of 120 min at 22 C, followed by a decrease in temperature to 4-5 C which was maintained for 120 min. Three samples of expired air and arterial blood were collected during the control period and four during acute cold exposure. During exposure to acute cold the dogs shivered constantly.

Thyroidectomized Dogs (THY)

Normal dogs weighing 9-17 kg were surgically thyroidectomized under morphine-pentothal anesthesia 4-5 weeks prior to the experiment. Completeness of the surgical procedure was judged by: gain in body weight, decrease in BMR, increase in plasma cholesterol in excess of 300 mg/100 ml, and an increase in low and very low density lipoproteins.

Pancreatectomized Dogs

Six dogs were surgically pancreatectomized under morphine-pentothal anesthesia 3-4 weeks prior to use. These dogs were maintained on Purina dog chow supplemented with meat, methionine, pancreatine and insulin (10-20 U/day).

General

Two days prior to the experiment indwelling arterial and venous catheters were placed in the carotid artery and jugular vein of each animal. Normal and THY dogs were fed Purina dog chow with meat. All experiments were performed after 18-24 hr of fasting. After a priming dose, palmitic-1-^{14}C acid bound to human albumin was infused intravenously at a constant rate throughout all the experiments reported here. The expired respiratory gases were collected in Douglas bags from a plastic hood fitted around each dog's head and analyzed for O_2 and CO_2 content by means of a calibrated Noyons diaferometer (2). $^{14}CO_2$ was trapped and measured by the methods of Fredrickson and Ono (5). Blood samples were taken immediately after the collection of air

TABLE I

FFA Metabolism in Normal, THY and Pancreatectomized Dogs[a]

Dogs	$\dot{V}O_2$, ml/kg/min	RQ	Plasma FFA, μEq/ml	FFA turnover, μEq/kg/min	FFA turnover oxidized, %	FFA oxidized, μEq/kg/min	CO_2 from plasma FFA, %	Plasma glucose, mg/100 ml	Blood lactic acid, mg/100 ml
Normal, $n = 39$	6.98 ±0.17	0.817 ±0.010	0.576 ±0.037	18.6 ±0.76	22.5 ±0.74	4.28 ±0.26	29.0 ±1.82	98.8 ±1.62	11.42 ±0.68
Thyroidectomized, $n = 15$	4.93 ±0.21	0.858 ±0.021	0.596 ±0.058	12.2 ±1.03	19.1 ±1.64	2.38 ±0.28	19.3 ±2.21	105.2 ±2.46	17.98 ±2.26
P values[b]	<0.001	N.S.	N.S.	<0.001	N.S.	<0.001	<0.01	<0.05	<0.01
Pancreatectomized,[c] $n = 6$	7.59 ±0.45	0.722 ±0.017	1.942 ±0.34	44.5 ±6.9	16.6 ±0.1	7.07 ±0.75	79.1 ±4.9	330.8 ±11.2	8.09 ±1.1

[a]Values are means ± SE, n = number of experiments.
[b]Comparison between normal and thyroidectomized dogs.
[c]Insulin withheld for 48 hr prior to the experiment.

TABLE II

FFA Metabolism in Normal Dogs during Exercise and Acute Cold Exposure

Dogs	Time, min	$\dot{V}O_2$, ml/kg/min	RQ	Plasma FFA, μEq/ml	FFA turnover, μEq/kg/min	FFA turnover oxidized, %	FFA oxidized, μEq/kg/min	CO_2 from plasma FFA, %	Blood lactic acid, mg/100 ml	Plasma glucose, mg/100 ml
A. FFA metabolism in normal dogs during exercise[a]										
					n = 12					
Trained (6)	0-60	45.4 ±1.0	0.818 ±0.012	0.76 ±0.06	51.0 ±2.6		37.5 ±2.6	38.5 ±2.8	16.6 ±0.8	
					n = 15					
	120-240	45.0 ±0.9	0.758 ±0.006	1.28 ±0.07	77.3 ±3.3		52.1 ±2.0	57.7 ±2.1	16.6 ±1.9	
	P values	N.S.	<0.001	<0.001	<0.001		<0.001	<0.001	N.S.	
					n = 25					
Untrained (12)	30-40	57.8 ±1.1	0.886 ±0.008	0.59 ±0.06	26.2 ±1.9		23.1 ±1.7	17.5 ±1.4	45.3 ±3.1	
	P values[b]	<0.001	<0.001	N.S.	<0.001		<0.001	<0.001	<0.001	
B. FFA metabolism in normal dogs during acute cold exposure[c]										
Before shivering at 22 C		6.97 ±0.39	0.801 ±0.013	0.605 ±0.091	17.6 ±2.14	22.1 ±1.1	3.85 ±0.37	24.3 ±1.8		99.6 ±4.0
During shivering at 4-5 C		10.04 ±0.28	0.747 ±0.015	1.018 ±0.052	28.0 ±0.92	32.7 ±1.4	9.12 ±0.42	45.9 ±2.4		97.5 ±6.2
	P values	<0.001	<0.05	<0.01	<0.01	<0.001	<0.001	<0.001		N.S.

[a]Values are means ± SE of n = number of determinations.
Number in parentheses, column 1 = number of experiments.
[b]Comparison of untrained dogs with trained running from 0-60 min.
[c]Values are means ± SE of five experiments.

and analyzed for FFA specific activity (FFA SA), plasma glucose and blood lactate as described in our previous work (3,6). As in our previous studies (7), the rates of mobilization and oxidation of plasma FFA were calculated from the FFA SA, CO_2SA, CO_2 output and the infusion rate of 1-^{14}C-palmitate according to the equation of Steele et al. (8). Control values shown in the tables are the averages of three to four samples taken during a rest period of 110-120 min.

Anaphylactic Shock

When a second experiment with albumin-bound palmitate was conducted 1 or 2 weeks later, four dogs experienced anaphylactic shock immediately after the priming dose of tracer. The symptoms were polypnea, dyspnea, urination, defecation and restlessness. During the first 120 min of the shock phase, air and blood samples were taken.

Infusion Studies

Sodium L (+) lactate (pH 7.4) was given intravenously to three normal dogs at a rate of 7-15 mg/kg min for 35-40 min. In another group of normal dogs, sodium nicotinate was infused at the rate of 1.5-7 mg/kg/min for 45 min. One animal experiencing anaphylactic shock received an infusion of norepinephrine at the rate of 0.5 μg/kg/min for 150 min.

RESULTS AND DISCUSSION

Table I summarizes experiments in 39 normal, 15 thyroidectomized and 6 pancreatectomized dogs under basal conditions. Normal dogs at a mean FFA level of 0.58 μEq/ml had an FFA turnover rate of 18.6 μEq/kg/min of which 22.5% was immediately oxidized, contributing 29% of the exhaled CO_2. As expected, THY dogs had a significantly lower O_2 consumption. The FFA level was 0.6 μEq/ml, the same as in normal dogs, but a significantly lower FFA turnover rate of 12.2 μEq/kg/min was found. The amount of FFA oxidized was only half of that in normal dogs. On the other hand, pancreatectomized dogs had a markedly elevated FFA level of 1.94 μEq/ml and a correspondingly high FFA turnover rate of 44.5 μEq/kg/min, 17% of which was oxidized, contributing up to 80% of the exhaled CO_2.

Although the results of these experiments clearly indicate that hormones have a profound effect on FFA metabolism, they suggest that metabolic rate is important too. Comparison of the data obtained in normal and pancreatectomized animals shows a direct relationship between plasma FFA level and turnover rate as shown in normal dogs by Issekutz et al. (2); but thyroidectomized animals with a lower metabolic rate had a decreased FFA turnover rate, despite an essentially normal FFA level. Furthermore no major differences were observed in the percentage of FFA turnover which was oxidized in the three groups studied. Thus the amount of FFA oxidized appears to depend on the turnover rate and, when animals with different metabolic rates are compared, to be independent of plasma FFA level. In other words, it would appear that not only the plasma FFA level but the amount of substrate released from adipose tissue and taken up by the whole system per unit of time is the important determining factor in the amount of FFA oxidized during resting conditions.

The effect of exercise on FFA metabolism in trained and untrained dogs is shown in Table IIA. Although the trained dogs exercised for 4 hr, data are shown only for the 1st hr and the last 2 hr. At the end of the 1st hr of exercise the FFA level increased from the resting mean level of 0.45 ± 0.03 μEq/ml to 0.76 ± 0.06 μEq/ml, while the FFA turnover rate had risen to 51 μEq/kg/min of which 75% was immediately oxidized, contributing 38% of the exhaled CO_2. During the last 2 hr of exercise, a further rise in FFA level and turnover rate was found and the oxidation of plasma FFA accounted for 58% of the exhaled CO_2, indicating a shift in metabolism toward fat oxidation. In contrast to the trained dogs, untrained animals showed a slight decrease in FFA level, from the resting value 0.8 ± 0.04 to 0.59 ± 0.06 μEq/ml during exercise. Although a larger percentage of the FFA turnover rate was immediately oxidized, only 18% of CO_2 was derived from plasma FFA oxidation. Paul and Issekutz (9) showed that the oxidation of plasma glucose accounts for not more than 15% of the caloric expenditure during exercise in trained and untrained dogs, and since the muscle probably stores only a limited amount of FFA in the form of triglyceride, this tissue must depend on extramuscular energy depots to perform work of long duration. Thus, in untrained dogs where enhanced rates of FFA mobilization did not occur, work was carried on mostly at the expense of stored carbohydrate and consequently was of short duration.

Results obtained from five experiments in normal dogs before and during acute cold exposure (shivering) are summarized in Table IIB. The values seen in the control period were essentially the same as those in the normal dogs presented in Table I. Acute cold exposure increased O_2 uptake to 10 ml/kg/min, while the FFA level rose from 0.61 to 1.02 μEq/ml. During the same period, the FFA turnover rate

TABLE III

FFA Metabolism in Dogs during Anaphylactic Shock

Condition or time	$\dot{V}O_2$, ml/kg/min	RQ	Plasma FFA, μEq/ml	FFA turnover, μEq/kg/min	FFA turnover oxidized, %	FFA oxidized, μEq/kg/min	CO_2 from plasma FFA, %	Blood lactic acid, mg/100 ml	Plasma glucose, mg/100 ml
A. FFA metabolism during anaphylactic shock									
Normal	6.64	0.881	0.151	5.4	8.9	0.48	3.1	54.6	87.8
Thy	4.05	1.030	0.195	5.7	6.7	0.38	3.5	54.3	89.9
Thy[a]	4.30	1.037	0.239	4.6	3.0	0.14	1.2	92.9	538.1
B. FFA metabolism during terminal state of anaphylactic shock									
Time, min									
60	4.91	1.030	0.281	5.65	2.9	0.166	1.38	94.8	507.4
90	3.69	1.043	0.197	3.51	3.0	0.104	1.02	91.0	568.8
120	1.02	0.814	0.158	2.81	0.5	0.013	0.59	95.8	661.0
C. Changes in FFA metabolism during anaphylactic shock and following norepinephrine infusion									
Conditions									
Control	6.59	0.881	0.752	21.0	19.9	4.18	27.3	11.9	105.6
Anaphylactic shock	6.35	1.007	0.587	10.9	7.2	0.79	4.6	33.9	131.1
Norepinephrine[b] infusion	6.16	0.890	0.976	17.1	10.3	1.76	12.4	19.7	101.8
D. FFA metabolism during rest and exercise under normal conditions and during anaphylactic shock[c]									
Conditions									
Control									
Rest	6.96	0.726	0.768	18.3	22.2	4.02	29.9	9.7	112.9
Exercise	40.2	0.755	0.701	36.3	70.8	25.7	32.0	23.9	108.0
Anaphylactic shock									
Rest	6.64	0.881	0.151	5.4	8.9	0.48	3.07	54.6	87.8
Exercise	45.8	0.944	0.220	16.6	29.0	3.34	2.96	80.4	95.7

[a]Terminal stage of anaphylactic shock.
[b]Norepinephrine infusion 0.5 μg/kg/min.
[c]Experiments conducted on the same dog.

rose from 18 to 28 μEq/kg/min, while the rate of plasma FFA oxidation increased from 3.9 to 9.1 μEq/kg/min. The percentage of respiratory CO_2 from plasma FFA oxidation doubled.

During the period of stress induced by acute cold exposure, several major changes occurred: (a) There was an increase in the plasma FFA level and turnover rate, and (b) the amount of FFA that was immediately oxidized increased significantly, i.e., by ca. 150%, while the metabolic rate increased ca. 50%. This would appear to answer the question so often raised whether the increase in FFA oxidation is proportional to the increase in energy expenditure due to acute cold exposure.

Table IIIA shows the changes that occurred during anaphylactic shock in one normal and two THY dogs. No change in O_2 consumption during shock was observed. In each of these experiments, an increased lactic acid level was found, especially during the terminal stage of shock seen in the third animal where the lactic acid rose to 93 mg/100 ml. Also the plasma glucose level in this animal increased strikingly to 538 mg/100 ml. The RQ was in the range of 0.88-1.03, suggesting that carbohydrate oxidation predominated.

In these dogs the plasma FFA levels and turnover rates were extremely low, as was the percentage of turnover immediately oxidized. Because of this the amount of FFA oxidized, as well as the percentage of respiratory CO_2 derived from plasma FFA oxidation, was negligible.

Such changes are even more dramatically shown by the data in Table IIIB which were obtained in one dog in the terminal stages of anaphylactic shock. In this animal, signs of anaphylactic shock were evident a few minutes after administration of the priming dose of albumin-bound palmitate-^{14}C and 120 min later the dog died. Data obtained during the last hour of the experiment are shown in the table. During this period, lactic acid and glucose levels were markedly elevated and O_2 consumption declined steadily. The already depressed FFA level, turnover rate and rate of oxidation also continued to decline.

Table IIIC compares FFA metabolism under three experimental conditions in the same dog: a control period, during anaphylactic shock and during anaphylactic shock and norepinephrine infusion. Although the degree of anaphylactic shock was mild, the lactic acid level rose to 34 mg/100 ml, and there was a slight decrease in plasma FFA level and turnover rate. A dramatic change was observed in the percentage of FFA turnover oxidized, which fell from 19.9 to 7.2%. This was further reflected in the amount of FFA oxidized which decreased from 4.2 to 0.8 μEq/kg/min. At the same time, the contribution of plasma FFA to the exhaled CO_2 dropped from 27% to 4.6%. A norepinephrine infusion of 0.5 μg/kg/min was then administered for 150 min. During this infusion, the FFA level and turnover rate increased two-fold. In spite of this the percentage of FFA turnover rate immediately oxidized remained low, in the range of 10%. Only 1.8 μEq/kg/min of FFA was oxidized accounting for 12% of the exhaled CO_2, which is only half of that found under control conditions. Thus, although norepinephrine was able to restore mobilization to normal levels in the shocked animal, oxidation remained low, implying an impairment of oxidative mechanisms.

The effect of exercise on FFA metabolism during anaphylactic shock in the same dog is shown in Table IIID. The table compares results obtained from the first 45 min of exercise under normal conditions with those from the first 35 min of exercise during anaphylactic shock, after which the dog was exhausted and refused to run farther. In both experiments exercise increased the oxygen consumption six- to seven-fold. In the control experiment the FFA level did not change during exercise, but FFA mobilization increased from 18 to 36 μEq/kg/min. Of the FFA turnover 70.8% was immediately oxidized, contributing 32% of the exhaled CO_2. The dog continued to exercise up to 90 min, during which time the FFA level rose to 2.5 μEq/ml and the FFA turnover rate increased to 95.9 μEq/kg/min. Under these conditions 70-80% of the energy requirement came from FFA oxidation. During anaphylactic shock the plasma FFA level and its rate of mobilization and oxidation were considerably depressed during both rest and exercise. Only 3% of the expired CO_2 derived from FFA oxidation during exercise, about one-tenth that found under control conditions. The high lactate level seen during anaphylactic shock increased even further during exercise to 80 mg/100 ml.

Table IV shows that normal dogs infused with nicotinic acid have a plasma FFA level and rate of mobilization significantly lower than those of control animals. Despite the fact that a similar percentage of FFA turnover is oxidized in both groups of animals, a significantly lower rate of turnover in the dogs infused with nicotinic acid resulted in a lower rate of oxidation, 2.1 μEq/kg/min, and a smaller contribution to total CO_2 output, 15.4%.

Results obtained in three dogs infused with sodium L (+) lactate (Table IV) show blood lactic acid levels comparable to those seen

during anaphylactic shock. Furthermore, during infusion, all measurements of FFA metabolism decreased by ca. 50%, except the percentage of FFA turnover immediately oxidized which remained the same.

After the infusion of nicotinic acid or lactic acid into normal animals, marked decreases in plasma FFA levels and rates of turnover were observed. These values are comparable to those seen in animals during anaphylactic shock. On the other hand, rates of FFA oxidation in the infused animals were considerably higher than those observed during anaphylactic shock and reinforce the suggestion previously made that an impairment in oxidative mechanisms may exist under these conditions.

Under normal conditions, carbohydrate and lipid, in the forms of glucose and FFA, respectively, are considered to be the major metabolic fuels from which the total caloric expenditure is derived. Only in extreme conditions and especially when carbohydrate supplies are depleted, does protein become a fuel of metabolic significance. The fuels of metabolic importance in shock induced by various means or following accidental or iatrogenic injury have yet to be determined quantitatively. This is so both for the acute or "ebb" phase and for the later or "flow" phase following shock or injury.

A reciprocal relationship between glucose and FFA oxidation has been repeatedly described, i.e., under conditions where rates of glucose oxidation are elevated, FFA oxidation is depressed. Our results, obtained during the acute phase of anaphylactic shock and suggesting a high rate of glucose oxidation coupled with depressed rates of FFA oxidation, are in agreement with this concept. Long et al. (10) have shown in man that glucose metabolism is altered following major surgery or infection. This alteration was indicated by an increased pool size, and rate of turnover and oxidation of glucose. Tissue anoxia, as a consequence of shock, has been shown to stimulate glucose uptake in experiments in vitro with rat diaphragm muscle. Randle and Smith (11) and Drucker and De Kiewiet (12) have demonstrated enhanced rates of glucose uptake and lactate production in diaphragm muscle removed from rats subjected to hemorrhagic shock.

Thus far, depressed rates of FFA mobilization or oxidation following injury or shock have not been conclusively demonstrated. Although Spitzer and Spitzer (13) have recently reported a decreased plasma FFA concentration in most dogs during hemorrhagic shock, others have shown enhanced rates of FFA mobilization after injury (Birke et al. [14]), burns (Birke et al. [15]) or fracture (Carlson and Liljedahl [16]). However the latter studies were performed many hours after the injury occurred and, in this sense, are not directly comparable to our experiments. From our studies it is apparent that soon after the onset of anaphylactic shock the mobilization and oxidation of plasma FFA are markedly decreased. The depressed rate of oxidation is apparently not simply related to the low rate of mobilization since dogs in anaphylactic shock, when exercised or infused with norepinephrine, continued to display lower oxidative rates compared to normal animals. Furthermore, rates of FFA oxidation in normal animals infused with nictotinic acid were higher than in animals in anaphylactic shock, despite similar plasma FFA levels and rates of mobilization.

Previously, Issekutz et al. (6) demonstrated that lactic acid inhibited FFA mobilization. Although lactic acidemia was consistently observed in animals in anaphylactic shock, infusions of this compound into normal animals failed to depress FFA oxidation to the same degree. It should be noted that the percentage of FFA turnover, which was immediately oxidized, was not altered by infusions of lactic or nicotinic acid into normal animals, but in animals during anaphylactic shock it was markedly decreased. Thus the depressed rate of FFA oxidation in these animals is the result of a decreased rate of FFA mobilization coupled with a smaller percentage of FFA turnover which was immediately oxidized. Others have also reported that substrate oxidation is affected by shock. Schumer and Sperling (17) emphasized that hypovoleamic shock leads to cell anoxia with a block in conversion of pyruvate to acetyl-CoA, a progressive tissue deficit in ATP and a buildup of lactic acid. Work of Stoner et al. (18) and Heath and Threlfall (19) indicated that the glucose entry into the glycolytic pathway and the oxidation in the tricarboxylic cycle were decreased, indicating interference at or near the citrate synthesis stage where oxaloacetate and acetyl-CoA are combined to form citrate. Stoner et al. (20) also showed that the oxidation of pyruvate and free fatty acids was inhibited equally during the first hours after release of tourniquets.

Diminished blood flow during shock, as found by Kovach et al. (21), could be an additional factor in suppressing FFA mobilization. Peterson et al. (22) found that an inadequate O_2 supply to the muscle leads to competition between pyruvic acid and dilydroxyacetone phosphate (DHAP) for $DPNH_2$, resulting in a striking accumulation of lactate and α-glycerophosphate (α-GP). The same inade-

TABLE IV

Effects of Nicotinic and Lactic Acids on FFA Metabolism in Normal Dogs

Condition	O_2, ml/kg/min	Plasma FFA, μEq/ml	FFA turnover, μEq/kg/min	FFA turnover oxidized, %	FFA oxidized, μEq/kg/min	CO_2 from plasma FFA, %	Blood lactic acid, mg/100 ml
				Nicotinic acid[a]			
Control	6.98	0.580	18.6	22.5	4.28	28.9	11.4
n = 39	±1.04	±0.237	±4.7	±4.6	±1.61	±11.4	±3.7
Ni Ac	6.28	0.200	10.6	19.7	2.12	15.4	14.2
n = 6	±0.48	±0.022	±1.3	±4.8	±0.70	±5.6	±3.5
P values	N.S.	<0.001	<0.001	N.S.	<0.01	<0.01	N.S.
				Lactic acid			
Dogs	Before lactate infusion						
A		0.54	15.1	21.4	3.4	19.9	9.0
B		0.69	14.1	39.0	5.5	39.0	10.3
C		0.81	21.4	39.7	8.5	40.0	17.0
	During lactate infusion						
A_1		0.37	11.0	16.4	1.8	10.4	53.7
B_1		0.36	9.0	37.8	3.4	23.9	66.2
C_1		0.28	9.0	40.0	3.6	17.1	109.0

[a]Values are means ± SE, n = number of experiments.

quate O_2 supply present during anaphylactic or other shock conditions may result in the inhibition of FFA mobilization from adipose depots due to an increase of α-GP. As a result of an inadequate O_2 supply, intracellular lactic acid concentration is obviously much higher during shock conditions than it is during lactic acid infusion, although in both cases plasma lactate concentrations are the same. Under these conditions an increase in α-GP supply could cause the reesterification of FFA, which would be reflected in the smaller percentage of FFA turnover being oxidized. It is of interest that the same process (accumulation of α-GP) may affect the two sites of FFA metabolism: (a) inhibiting FFA mobilization from the adipose depots, and (b) suppressing FFA oxidation in tissues using it as fuel.

On the basis of these experiments, it is evident that not only FFA mobilization but also FFA utilization is affected by the different metabolic states induced by stress. During physiological stress, FFA mobilization increases. The amount of FFA oxidized depends on the amount mobilized and on the metabolic rate of the tissue using FFA as an energy substrate.

During the stress of anaphylactic shock, FFA mobilization is inhibited and FFA utilization is depressed. Raising the FFA mobilization to a normal range with norepinephrine or increasing the energy demand with exercise has no effect on the FFA oxidation rate which remains low.

The rate of energy substrate delivery to the tissues by the circulation and the oxidative machinery in these tissues are of equal importance for our biological survival under basal conditions. Under stress these factors play an even more significant role.

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REFERENCES

1. Armstrong, D.T., R. Steele, N. Altszuler, A. Dunn, J.S. Bishop and R.C. De Bodo, Amer. J. Physiol. 201:9 (1961).
2. Issekutz, B., Jr., H.I. Miller, P. Paul and K. Rodahl, Ibid. 207:583 (1964).
3. Paul, P., J. Appl. Physiol. 28:127 (1970).
4. Paul P., in "Advances in Experimental Medicine and Biology," Vol. 11, Edited by B. Pernow and B. Saltin, Plenum Press, New York, 1971 p. 225.
5. Fredrickson, C.S., and R. Ono, J. Lab. Clin. Med. 51:147 (1958).
6. Issekutz, B., Jr., H.I. Miller, P. Paul and K. Rodahl, Amer. J. Physiol. 209:1137 (1965).
7. Issekutz, B., Jr., P. Paul and H.I. Miller, J. Appl. Physiol. 213:857 (1967).
8. Steele, R., N. Altszuler, J.S. Wall, A. Dunn and R.C. De Bodo, Amer. J. Physiol. 196:221 (1959).
9. Paul, P., and B. Issekutz, Jr., J. Appl. Physiol. 22:615 (1967).
10. Long, C.L., J.L. Spencer, J.M. Kinney and J.W. Geiger, Ibid. 31:110 (1971).
11. Randle, P.J., and G.H. Smith, Biochem. J. 70:470 (1958).
12. Drucker, W.R., and J.C. De Kiewiet, Amer. J. Physiol. 206:317 (1964).
13. Spitzer, J.J., and J.A. Spitzer, Amer. J. Appl. Physiol. 222:101 (1972).
14. Birke, G., L.A. Carlson and S.-O. Liljedahl, Quart. J. Exp. Physiol. 39:1 (1954).
15. Birke, G., L.A. Carlson and S.-O. Liljedahl, Acta Med. Scand. 178:337 (1965).
16. Carlson, L.A., and S.-O. Liljedahl, Ibid. 173:25 (1963).
17. Schumer, W., and R. Sperling, JAMA 205:75 (1960).
18. Stoner, H.B., D.F. Heath and O.M. Collings, Biochem. J. 76:135 (1960).
19. Heath, D.F., and C.J. Threlfall, Ibid. 110:337 (1968).
20. Stoner, H.B., D.F. Heath, C.J. Threlfall and M.M. Ashby, in "Intermedes Proceedings 1967; Combined Injuries and Shock," Edited by B. Schilot and L. Thoren, Försvarets Forskningsanstalt, Stockholm, 1968, p. 115.
21. Kovach, A.G.B., S. Rosell, P. Sandor, E. Koltay, M. Hamori and E. Kovach, Naunyn-Schmiedebergs Arch. Pharmak. 268:140 (1971).
22. Peterson, R.D., D. Gaudin, R.M. Bocer and C.H. Beatty, Amer. J. Physiol. 206:599 (1964).

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SHORT COMMUNICATIONS

Enhancement of Liver Microsomal Acyl CoA-Lysophospholipid Acyltransferase Activity in Pyridoxine-Deficient Rats

ABSTRACT

In rats deficient in pyridoxine and essential fatty acids, liver phospholipids contained less arachidonic acid and more oleic and eicosatrienoic acids than those from animals only deficient in essential fatty acids. This pattern persisted after the animals were supplemented with linoleate for 6 days. Liver oleyl and arachidonyl CoA-lysophospholipid acyltransferase activities were significantly higher in pyridoxine-deficient animals. Supplementation with linoleate for 6 days resulted in a marked increase in arachidonyl CoA-acyltransferase activity in pyridoxine-deficient rats but a relatively small increase in the supplemented animals. Differences in fatty acid composition between pyridoxine-deficient and supplemented rats can not be ascribed to lower liver transacylase activity in the deficient animals.

The apparent involvement of pyridoxine in polyunsaturated fatty acid metabolism was first reported by Witten and Holman (1), who found that pyridoxine- and essential fatty acid (EFA)-deficient rats accumulated less arachidonic acid in their tissues when supplemented with linoleate than did animals deficient in EFA but receiving adequate dietary vitamin B_6. A similar deficit in tissue (*n*-3)-polyunsaturated acids was noted in pyridoxine-deficient rats supplemented with linolenate. Although Witten and Holman believed that the conversion of linoleate to arachidonate was impaired in B_6-deficient animals, this concept has not been supported by other researchers in this field (2,3). It is possible that pyridoxine is involved in the metabolism of arachidonic acid rather than in its synthesis from linoleate. The present study was undertaken to test one aspect of this hypothesis, namely that pyridoxine deficiency impairs incorporation of arachidonate into tissue phosphatides.

Male weanling rats of the Wistar strain were maintained for six weeks on semipurified diets containing glucose (60%), casein (vitamin-free, 20%), hydrogenated coconut oil (10%), cellulose (5%), minerals and vitamins. Pyridoxine was omitted from the diet of one group of rats ($-B_6$,-EFA); a second group received the B_6-supplemented diet ($+B_6$,-EFA) ad libitum and a third group received the B_6-supplemented diet ($+B_6$,-EFA) pair-fed to the pyridoxine-deficient group. After 6 weeks half of the animals from each group were sacrificed, and the livers were subjected to lipid analyses and enzyme assay. The remaining animals received 150 mg ethyl linoleate daily by stomach tube for 6 days; they were sacrificed and the livers subjected to the analytical procedures. Liver lipids were extracted with chloroform-methanol 2:1 and fractionated by thin layer chromatography on Silica Gel G developed in petroleum ether, ether, acetic acid 90:10:1 v/v. The total phospholipid fraction (including sphingomyelin), which remained at the origin, was extracted from the gel with chloroform-methanol 1:1, transesterified with boron fluoride-methanol (4) and analyzed by gas liquid chromatography. A second portion of liver was homogenized at 4 C in 0.05M Tris-HCl buffer (pH 7.6), containing 0.35 M sucrose, 0.025 M KCl and 0.01M MgCl, and the microsomal fraction was isolated by centrifugation at 105,000 g (5). Protein was determined by the procedure of Lowry et al. (6). Acyl CoA-lysophospholipid acyltransferase was assayed by the method of Lands and Hart (7) using oleyl or arachidonyl CoA as substrate.

After 6 weeks on the experimental diets, the proportion of arachidonic acid in the liver phospholipids was significantly lower in the B_6-deficient animals (Table I) than in either of the control groups, confirming data previously reported (8). Eicosatrienoate was significantly higher in the pyridoxine-deficient group. These differences can be attributed to the lack of pyridoxine in the diet rather than the depressed food consumption, since the B_6-deficient animals differed significantly from the pair-fed controls, which did not differ from the ad libitum controls. It is apparent from the low

TABLE I

Effect of Pyridoxine Deficiency on Major Unsaturated Fatty Acids of Rat Liver Phospholipids

Acid	Treatment					
	-B_6,-EFA ad libitum		+B_6,-EFA pair-fed control		+B_6,-EFA ad libitum control	
	No 18:2 supplementation	18:2 supplemented[a]	No 18:2 supplementation	18:2 supplemented	No 18:2 supplementation	18:2 supplemented
	% Total fatty acids[b]					
18:1	21.8●	8.9■	20.7●	11.7▲	17.9★	10.8▲
18:2	4.4●	8.4■	5.3●	11.3▲	4.3●	10.8▲
20:3(*n*-9)	19.8●	9.9■	15.4▲	6.2★	16.1▲	6.2★
20:4(*n*-6)	6.9●	16.7■	9.0▲	22.8★	9.8▲	24.2★

[a]After 6 weeks on experimental diets, rats received 150 mg ethyl linoleate daily for 6 days.

[b]Each value represents mean of 5 samples. Means in each row followed by same symbol are not significantly different (P>0.05) by analysis of variance and Duncan's multiple range test.

TABLE II

Activities of Liver Microsomal Acyl CoA-Lysophospholipid Acyltransferases in Rats Depleted of Pyridoxine and EFA

Enzyme[a]	Treatment					
	-B_6,-EFA ad libitum		+B_6,-EFA pair-fed control		+B_6,-EFA ad libitum control	
	No 18:2 supplementation	18:2 supplemented[b]	No 18:2 supplementation	18:2 supplemented	No 18:2 supplementation	18:2 supplemented
	Activity,[c] mμmol product min/mg microsomal protein					
OCLAT	41.4●	49.1■	40.1●	30.3▲	32.9▲	30.0▲
ACLAT	114.6★	211.8◆	106.9★	83.1◆	92.9◆	117.9★

[a]OCLAT, oleyl CoA-lysophospholipid acyltransferase; ACLAT, arachidonyl CoA-lysophospholipid acyltransferase.

[b]After 6 weeks on experimental diets, rats received 150 mg ethyl linoleate daily for 6 days.

[c]Each value represents mean of five samples. Means in each row followed by same symbol are not significantly different (P>0.05) by analysis of variance and Duncan's multiple range test.

linoleate and arachidonate and high oleate and 20:3(*n*-9) levels that the animals were EFA-deficient. Supplementation with dietary linoleate for 6 days partially alleviated the symptoms of EFA deficiency, decreasing the oleate and 20:3(*n*-9) and increasing the 18:2 and 20:4(*n*-6). The levels of all four acids differed significantly between the B_6-deficient and control animals after supplementation with linoleate.

The activities of oleyl CoA-lysophospholipid acyltransferase (OCLAT) and arachidonyl CoA-lysophospholipid acyltransferase (ACLAT) are summarized in Table II. Before supplementation with dietary linoleate, liver microsomal OCLAT and ACLAT were significantly higher in the B_6-deficient and pair-fed controls than in the B_6-supplemented, ad libitum controls. After supplementation with linoleate, there was a slight increase in OCLAT activity and a very marked increase in ACLAT activity in the pyridoxine-deficient group. In the ad libitum controls, a small but significant increase in ACLAT activity was noted, whereas in the pair-fed controls the activity of both enzymes decreased significantly.

The results do not support the hypothesis that the differences in fatty acid composition between normal and pyridoxine-deficient rats are attributable to lower transacylase activities in the deficient animals. Although phospholipid arachidonate was significantly lower in the livers from B_6-deficient rats, ACLAT activity was significantly higher. Coniglio et al. (9) reported a greater oxidation of 1-^{14}C-arachidonate in pyridoxine deficiency and suggested that the turnover of arachidonate was greater in these animals. The higher transacylase activity, coupled with the lower tissue phospholipid arachidonate in the B_6-deficient animals in the current study, is consistent with this concept of increased arachidonate turnover. Whether the effect of vitamin B_6 is at the lipolytic stage or at the oxidation stage is open to speculation. It is interesting to note, however, that several enzymes involved in fat metabolism have been reported to increase in activity in pyridoxine-deficient animals (10).

On the basis of the similarities in OCLAT and ACLAT activities between the B_6-deficient and pair-fed controls prior to linoleate supplementation, the higher transacylase activities in the deficient rats may be attributed to inanition accompanying the vitamin deficiency. However these two groups reacted quite differently when supplemented with linoleate, the enzyme activities increasing in the deficient group but decreasng in the pair-fed group. Because of the low feed intake, the pair-fed controls were essentially meal-eaters rather than nibblers. Such a shift in feeding pattern does affect tissue enzyme activities (10), and the results obtained with the pair-fed controls are difficult to interpret. It is interesting to note that linoleate supplementation stimulated both OCLAT and ACLAT activity in the deficient rats, although liver oleic acid levels declined as a result of this supplementation. It is possible that stimulation of transacylase activity is mediated via a feedback mechanism initiated by a turnover of fatty acid in the tissue rather than by a simple substrate induction. This speculation warrants further investigation but is consistent with the concept of greater turnover of fatty acids in pyridoxine-deficient animals.

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REFERENCES

1. Witten, P.W., and R.T. Holman, Arch. Biochem. Biophys. 41:266 (1952).
2. Kirschmann, J.C., and J.G. Coniglio, J. Biol. Chem. 236:2200 (1961).
3. Swell, L., M.D. Law, P.E. Schools and C.R. Treadwell, J. Nutr. 74:148 (1961).
4. Morrsion, W.R., and L.M. Smith, J. Lipid Res. 5:600 (1964).
5. Tata, J.R., in "Subcellular Components, Preparation and Fractionation," Edited by G.D. Birnie and S.M. Fox, Butterworths, London, 1969, p. 87.
6. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193:265 (1951).
7. Lands, W.E.M., and P. Hart, Ibid. 240:1905 (1965).
8. Scheier, G., and M.A. Williams, Biochem. J. 92:422 (1964).
9. Coniglio, J.G., F.P. Culp and A. Goswami, Lipids 2:484 (1967).
10. Sabo, D.J., R.P. Francesconi and S.N. Gershoff, J. Nutr. 101:29 (1971).

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Hepatic Galactosylceramide in Globoid Cell Leukodystrophy (Krabbe's Disease)

ABSTRACT

Glycosphingolipids were isolated from the liver of four patients with globoid cell leukodystrophy and compared to those from normal liver. The ratio of glucosylceramide to galactosylceramide was 0.8, 1.2, 1.3 and 7.5 in the four cases studied, compared to a value of greater than 10 in liver from control patients of the same age and sex. In addition to this accumulation of galactosylceramide, all four livers showed an elevated level of sulfatide, and in one case trihexosylceramide and globoside were also markedly elevated.

Globoid cell leukodystrophy (GCLD) is a fatal inherited disorder of glycosphingolipid metabolism that can be biochemically characterized by an almost total deficiency of galactosylceramide-β-D-galactosyl hydrolase (1). In contrast to many glycosphingolipid neurovisceral storage diseases, there is no gross storage of material (galactosylceramide [GL-1b]) in the brain. The accumulated GL-1b is specifically localized in "globoid cells," and the low overall level of GL-1b has been attributed to the early cessation of myelination caused by the death of the oligodendroglial cells (2). Galactosylceramide is only found in significant amounts in brain (especially white matter) (3), kidney (4), intestine (5), and pancreas (6), of normal patients, being virtually absent from liver and spleen. Suzuki (4) has shown that renal cerebroside levels are not markedly elevated in this disease (4), despite the almost complete absence of enzymatic activity in this organ. This study was carried out to see if accumulation of galactosylceramide could be detected in liver, and a marked elevation was found in three out of the four cases studied.

Liver samples from four patients with globoid cell leukodystrophy and controls were frozen at -40 C prior to analysis. Liver from three of the four patients was assayed for GL-1b β-galactosidase activity (7) and found to be markedly deficient (Table I). Galactosylceramide specifically labeled at C-6 of galactose was obtained from N. Radin, University of Michigan. All patients exhibited the typical clinical picture of the GCLD phenotype. Fibroblasts from the twin siblings of the fourth patient (S. Ho.) were shown by Suzuki and Suzuki (1) to exhibit the characteristic GCLD enzymic deficiency; in these two cases, onset was not until 4 years of age (B. Ho. brain at autopsy [age 6] contained typical globoid cells, but S. Ho. is still alive in decerebrate condition [14 years]). Glycosphingolipids were isolated as described previously (5), and quantitative estimations were carried out by gas liquid chromatography of the trimethylsilyl methyl glycosides derived from acid methanolysis of the glycolipids (8). All analyses were carried out in duplicate, and the thin layer chromatograms of GCLD liver glycolipids were characterized by the presence of two bands in the monohexosyl region, the upper band containing mainly glucosylceramide and the lower band mainly galactosylceramide (9).

In three of the four cases studied a marked elevation of GL-1b was found (Table II), and in two of these cases it was accompanied by an elevation of lactosylceramide (GL-2a). This latter finding is interesting in view of the report by McCluer and Evans of the accumulation of GL-2a in GCLD brain (10). Examination of formalin-fixed brain from case B. Ho. did not reveal an abnormal GL-2a level, in agreement with Eto and Suzuki (3). In one of the four cases (Ri), trihexosylceramide (GL-3) and glo-

TABLE I

Enzymic Diagnosis of Globoid Cell Leukodystrophy in Liver

	Per cent normal activity		
Patient	Galactosylceramide β-D-galactosidase	*p*-Nitrophenyl β-D-galactoside β-D-galactosidase (pH 4.0)	*p*-Nitrophenyl β-D-galactoside β-D-galactosidase (pH 5.0)
Ro	0.4	74	110
Ri	1.5	104	99
He	0.5	84	124
C.B1[a]	390.0	9	62

[a]Patient with G_{M1}-gangliosidosis type II.

TABLE II

Hepatic Glycosphingolipids in Globoid Cell Leukodystrophy and Controls

	Patient				
	Ro	Ri	B. Ho	He	Control
	Age of onset, month				
	3	6-9	48	6	---
	Age of death, month				
	9	15	72	15	12-72
Component	μmol/100 g fresh wt				
GL-1a (Glucosylceramide)	3.7	9.4	5.0	6.0	6.0
GL-1b (Galactosylceramide)	2.9	7.7	6.0	0.8	<0.5
GL-2a (Lactosylceramide)	10.4	4.1	10.0[a]	12.0	6.0
GL-1bS (Sulfatide)	1.5	10.6	3.6	2.5	<0.5
GL-3 (Trihexosylceramide)	3.8	14.9	4.0	1.5	3.0
GL-4 (Globoside)	2.4	25.0	4.0	1.4	2.0
G_{M3} (Hematoside)	19.0	17.0	14.0[a]	15.0	18.0

[a]The G_{M3} level is low, suggesting some decomposition to lactosylceramide during prolonged storage.

boside (GL-4) were also elevated. Liver from this patient contained the highest level of sulfatide (GL-1bS) found in any of the GCLD livers, but GL-2a was not elevated.

Although GCLD does not result in any readily apparent hepatic structural changes (11), it is evident from these studies that there is a characteristic accumulation of GL-1b which may be of diagnostic value. The enzymic assay of Suzuki and Suzuki (1,2) is obviously the most reliable test (requires the natural substrate specifically labeled in the galactose moiety) and can be carried out with leukocytes or serum. Since both the enzymic and chemical abnormality can be detected in liver, a liver biopsy appears preferable to a brain biopsy in cases where further confirmation of the disease is required. Examination of urine sediment (12) from two cases of GCLD showed a normal glycolipid content with glucosylceramide the major monohexosylceramide, and the report of Suzuki (4) was confirmed by the finding of normal levels in kidney from patient B. Ho. Similarly, fibroblasts which show the enzymic defect (1) do not accumulate GL-1b (9). Suzuki (4) has offered the plausible explanation that the residual enzymic activity (5%) in kidney is sufficient to catabolize the small amount of GL-1b found in this tissue. However the finding of GL-1b in liver is in agreement with the concept of the liver as a storage organ, and the value of liver glycolipid analyses in the diagnosis of sphingolipidoses has been amply demonstrated (13), including a recent report (14) of the accumulation of sulfatide (GL-1bS) in liver from patients with an analogous white matter disease, metachromatic leukodystrophy. Since analyses of glycolipid levels in livers from patients with other sphingolipidoses have been extremely consistent (5,13) one can perhaps interpret the variation seen in GCLD as resulting from genetic heterogeneity.

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REFERENCES

1. Suzuki, Y., and K. Suzuki, Science 171:73 (1971).
2. Suzuki, K., and Y. Suzuki, Proc. Nat. Acad. Sci. 66:302 (1970).
3. Eto, Y., and K. Suzuki, J. Neurochem. 18:503 (1971).
4. Suzuki, K., Lipids 6:433 (1971).
5. Dawson, G., J. Lipid Res. 13:207 (1972).
6. Dawson, G., Fed. Proc., In press (1973).
7. Austin, J., K. Suzuki, D. Armstrong, R.O. Brady, B.K. Bachhawat, J. Schlenker and D. Stumpf, Arch. Neurol. 23:502 (1970).
8. Clamp, J.R., G. Dawson and L. Hough, Biochim. Biophys. Acta 148:342 (1967).
9. Dawson, G., R. Matalon and A. Dorfman, J. Biol.

Chem. 247:5951 (1972).
10. Evans, J.E., and R.H. McCluer, J. Neurochem. 16:1393 (1969).
11. Yunis, E.J., and R.E. Lee, Lab. Invest. 21:415 (1969).
12. Desnick, R.J., G. Dawson, S.J. Desnick, C.C. Sweeley and W. Krivit, New Engl. J. Med. 284:739 (1971).
13. Dawson, G., in "Sphingolipids, Sphingolipidoses and Allied Disorders," Edited by B.W. Volk and S.M. Aronson, Plenum Press, New York, 1972, p. 395.
14. Moser, H., in "The Metabolic Basis of Inherited Disease," Edited by J.B. Stanbury, J.B. Wyngaarden and D.S. Fredrickson, McGraw-Hill, New York, 1972, p. 688.

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In Vivo Incorporation of ^{3}H Arachidonic Acid and ^{14}C Linoleic Acid into Liver Lipids from Essential Fatty Acid-Deficient Rats

ABSTRACT

Essential fatty acid (EFA)-deficient rats were injected intraportally with a labeled solution containing ^{3}H arachidonic acid and ^{14}C-linoleic acid during a 1 min period. Livers were quickly frozen, pulverized, and the lipids extracted and fractioned by thin layer chromatography. The incorporation of ^{3}H and ^{14}C into liver lipids was measured, and the per cent distribution of radioactivity into the different lipid fractions determined and compared with those previously obtained from normal rats. In contrast with normal rats, ca. 70% of the ^{3}H arachidonic acid and ^{14}C-linoleic acid incorporated into total lipids from EFA-deficient rats was recovered in the phospholipid fraction. From the results of this experiment, it is suggested that a more active deacylation-reacylation cycle in EFA-deficiency could be responsible for this increase.

The structural and functional alterations of essential fatty acid EFA-deficient mammalian tissues could be explained through a change in the fatty acid composition of the phospholipids of their membranes, with special reference to the arachidonic acid content of these lipoprotein structures, since this fatty acid, according to Collins (1), acts as a modulator of phospholipid metabolism.

We have recently studied the in vivo incorporation of ^{14}C-linoleic acid into liver lipids from EFA-deficient rats (2). From the results of these studies, it became evident that the incorporation of ^{14}C-linoleic acid is directed predominantly to the phospholipid fractions in the EFA-deficient rats. Since arachidonic acid is considered to be the principal EFA (3), it was of interest to study the simultaneous incorporation of this acid and its precursor, linoleic acid, into liver lipids from EFA-deficient rats.

Weanling male Wistar rats maintained during 120 days on a semisynthetic fat-free diet (4) were used. They were injected with 0.2 ml labeled fatty acids-rat serum complex solution (5) containing 10 μC 1-^{14}C linoleic acid (Radiochemical Centre, Amersham, England) (52.9 μC/mmole) and 5 μC arachidonic acid 8,9,11,12,14,15- ^{3}H (New England Nuclear, U.S.) (5-10 C/mmole) into the exposed portal vein during a 1 min period, under ether anesthesia. After the injection, the livers were removed and quickly frozen with liquid N_2, pulverized, and the powder extracted with chloroform-methanol 2:1 v/v (6). The lipids from the extract were fractioned by thin layer chromatography (7,8), and the lipid fractions on the silica transesterified (9). The fatty acid methyl esters were assayed for radioactivity in a Packard Tri-Carb Scintillation spectrometer. Aliquots of total lipids from the original extracts were transesterified and the fatty acid methyl ester chromatographied on $AgNO_3$-impregnated silica gel plates (19), scraped into vials and counted directly.

The differences between the total uptake and distribution of ^{14}C-linoleic acid and ^{3}H-arachidonic acid in rat liver lipids appear in Table I. Data from a previously published study using normal rats is included. Despite the rather similar relative incorporation of ^{3}H-arachidonic acid in 1,2-diacylglycerol fractions from both groups of rats, and coincident with a fall in the radioactivity incorporated into neutral lipids

Chem. 247:5951 (1972).
10. Evans, J.E., and R.H. McCluer, J. Neurochem. 16:1393 (1969).
11. Yunis, E.J., and R.E. Lee, Lab. Invest. 21:415 (1969).
12. Desnick, R.J., G. Dawson, S.J. Desnick, C.C. Sweeley and W. Krivit, New Engl. J. Med. 284:739 (1971).
13. Dawson, G., in "Sphingolipids, Sphingolipidoses and Allied Disorders," Edited by B.W. Volk and S.M. Aronson, Plenum Press, New York, 1972, p. 395.
14. Moser, H., in "The Metabolic Basis of Inherited Disease," Edited by J.B. Stanbury, J.B. Wyngaarden and D.S. Fredrickson, McGraw-Hill, New York, 1972, p. 688.

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Weanling male Wistar rats maintained during 120 days on a semisynthetic fat-free diet (4) were used. They were injected with 0.2 ml labeled fatty acids-rat serum complex solution (5) containing 10 μC 1-^{14}C linoleic acid (Radiochemical Centre, Amersham, England) (52.9 μC/mmole) and 5 μC arachidonic acid 8,9,11,12,14,15- ^{3}H (New England Nuclear, U.S.) (5-10 C/mmole) into the exposed portal vein during a 1 min period, under ether anesthesia. After the injection, the livers were removed and quickly frozen with liquid N_2, pulverized, and the powder extracted with chloroform-methanol 2:1 v/v (6). The lipids from the extract were fractioned by thin layer chromatography (7,8), and the lipid fractions on the silica transesterified (9). The fatty acid methyl esters were assayed for radioactivity in a Packard Tri-Carb Scintillation spectrometer. Aliquots of total lipids from the original extracts were transesterified and the fatty acid methyl ester chromatographied on $AgNO_3$-impregnated silica gel plates (19), scraped into vials and counted directly.

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from EFA-deficient rats, there was a statistically significant increase in the amounts of ^{3}H-arachidonic acid and ^{14}C-linoleic acid incorporated into phospholipids from EFA-deficient rats as compared to normal rats.

At least two different pathways are thought to be of importance for the incorporation of long chain fatty acids into liver phospholipids: one involves de novo synthesis via phosphatidic acid (11); the other depends on the acylation of lysophosphoglycerides (12).

On the basis of in vitro studies in tissues from normal rats (13,14), it has been claimed that the phosphatidic acid pathway is predominantly concerned with the formation of the monoenoic and dienoic species, whereas the more polyunsaturated molecules arise via acylation of endogenous lysophosphatides. These observations could explain the higher incorporation of ^{14}C-linoleic acid than ^{3}H-arachidonic acid in the 1,2-diacylglycerol fraction, the "key" intermediate for de novo synthesis of phospholipids. The different pattern of behavior of linoleic acid observed in EFA-deficient rats seems to indicate that this acid could be incorporated into the phospholipid molecules mainly via the deacylation-reacylation cycle. The decrease in the amounts of linoleic acid incorporated into the 1,2-diacylglycerol fraction, observed in the EFA-deficient rats, supports this interpretation.

Since the stability of phospholipids may depend on their content of arachidonic acid (1), the lack of this acid in EFA deficiency could promote the hydrolysis of these molecules and the formation of lysoderivatives, which are acceptors for fatty acids in the resynthesis of phospholipids (15). This finding and the known high affinity of the acyl-transferase for polyunsaturated fatty acids (16-18) would explain the rapid enrichment in ^{14}C-linoleic acid and ^{3}H-arachidonic acid of the phospholipid fractions from EFA-deficient rats.

On the other hand, triglycerides show a significantly smaller incorporation of both acids in the EFA-deficient rats compared with controls. This could be due to the lack of linoleic and arachidonic acids in the endogenous acyl-CoA pool, since the 3 position of the triglycerides may be a reflection of the composition of this pool (19).

The distribution of radioactivity in the $AgNO_3$-impregnated silica gel plates showed that ca. 94% of ^{14}C counts was recovered in the dienoic fractions and 96% of ^{3}H counts in the tetraenoic fraction.

TABLE I

Per Cent of Total Radioactivity Recovered from ^{3}H Arachidonic and ^{14}C Linoleic Acid Incorporated into Lipid Fractions

Experimental group	Per cent distribution of radioactivity[a]							
	1,2-Diacylglycerol		Triacylglycerol		3-*sn*-Phosphatidylcholine		3-*sn*-Phosphatidylethanolamine	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Normal[b,c] (5)	9.6 ±[d] 1.3	26.7 ± 3.9	31.8 ± 1.4	35.6 ± 4.4	33.6 ± 6.5	20.4 ± 2.7	10.5 ± 1.4	4.0 ± 0.8
	N.S.[e]	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
Essential fatty acid (8)	7.2 ± 2.5	11.9 ± 2.3	10.9 ± 2.3	17.5 ± 2.8	50.7 ± 5.9	53.9 ± 4.7	18.3 ± 3.6	15.0 ± 4.5

[a]Data are the standard deviations of the means.
[b]Numbers in parentheses indicate the number of animals in each group.
[c]Reference 5.
[d]Probability (P) values are related to essential fatty acid-deficient group.
[e]N.S. = not significant.

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REFERENCES

1. Collins, F.D., Biochim. Biophys. Acta 9:289 (1962).
2. Mercuri, O., and M.E. De Tomás, Lipids 6:858 (1971).
3. Mohrhauer, H., and R.T. Holman, J. Lip. Res. 4:151 (1963).
4. Peluffo, R.O., R.R. Brenner and O. Mercuri, J. Nutr. 81:110 (1963).
5. Mercuri, O., and M.E. De Tomás, Lipids 6:203 (1971).
6. Folch-Pi, J., M. Lees and G.H. Sloane-Stanley, J. Biol. Chem. 226:487 (1957).
7. Wagner, W., L. Horhamer and P. Wolff, Biochem. Z. 335:175 (1961).
8. Malins, D.C., and H.K. Mangold, JAOCS 37:576 (1960).
9. Stoffel, W., F. Chu and E.H. Ahrens, Anal. Chem. 31:307 (1959).
10. De Tomás, M.E., and R.R. Brenner, An. As. Quim. Argentina 52:253 (1964).
11. Kennedy, E.P., Fed. Proc. 16:847 (1957).
12. Lands, W.E.M., J. Biol. Chem. 235:2233 (1960).
13. Hill, E.E., D.R. Husbands and W.E.M. Lands, Ibid. 243:4440 (1968).
14. Van Golde, L.M.G., G.L. Scherphof and L.L.M. Van Deenen, Biochim. Biophys. Acta 176:635 (1969).
15. Stein, O., and Y. Stein, Ibid. 84:621 (1964).
16. Van Den Bosch, W.M. and L.L.M. Van Deenen, Ibid. 106:326 (1965).
17. Stoffel, W., H.G. Schiefer and G.D. Wolf. Hoppe-Seyler's Z. Phys. Chem. 347:102 (1966).
18. Stoffel, W., M.E. De Tomás and H.G. Schiefer, Ibid. 348:882 (1967).
19. Slakey, S.P., and W.E.M. Lands, Lipids 3:30 (1968).

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Cholestanol and Plant Sterols in Pigeon Skin

ABSTRACT

Cholestanol (4.6%) and plant sterols (0.2%) have been demonstrated, for the first time, in avian skin by argentation and gas liquid chromatography. In contrast to results of previous studies with rat and human skin, cholestanol represented a significant amount in the pigeon. The proportion of campesterol was always higher than that of β-sitosterol.

Studies of the skin of mammals (1-3) demonstrated numerous sterols in the skin, which has become recognized as a route of sterol excretion. Little is known, however, of the nature of sterols in avian skin. The objects of this study of skin sterols of the White Carneau pigeon were two-fold: (a) to determine the amount of cholestanol (5α-cholestan-3β-ol), since a significant amount of this stanol is excreted in the feces of this species (4); and (b) to determine the nature of plant sterols, if any, in the skin. This was of particular interest in view of previous observations (5) that plasma concentration and intestinal uptake of campesterol are higher than those of β-sitosterol.

MATERIALS AND METHODS

Skin samples were obtained from 2-year-old White Carneau pigeons (Palmetto Pigeon Plant, Sumter, S.C.). Skin samples were weighed and minced, and total lipids were extracted as described by Folch et al. (6). The extracts were saponified, and free sterols were extracted as described previously (7). The unsaponifiable fraction was subjected to thin layer chromatography using the solvent system, heptane-isopropyl ether-acetic acid 65:40:4 v/v to separate cholesterol and plant sterols from 4-methyl sterols and lanosterol derivatives. Cholestanol was separated from Δ^5-sterols by argentation chromatography using the solvent system, chloroform-methanol-acetic acid 100:1:0.2 v/v (7). After elution of different sterol fractions from the silica gel, the sterols were identified and quantitated by gas liquid chromatography on 3.8% W-98 columns (4,5).

RESULTS AND DISCUSSION

During thin layer chromatography of the unsaponifiable fraction, a major band corresponding to cholesterol was noticed. Unlike the

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REFERENCES

1. Collins, F.D., Biochim. Biophys. Acta 9:289 (1962).
2. Mercuri, O., and M.E. De Tomás, Lipids 6:858 (1971).
3. Mohrhauer, H., and R.T. Holman, J. Lip. Res. 4:151 (1963).
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8. Malins, D.C., and H.K. Mangold, JAOCS 37:576 (1960).
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13. Hill, E.E., D.R. Husbands and W.E.M. Lands, Ibid. 243:4440 (1968).
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15. Stein, O., and Y. Stein, Ibid. 84:621 (1964).
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17. Stoffel, W., H.G. Schiefer and G.D. Wolf. Hoppe-Seyler's Z. Phys. Chem. 347:102 (1966).
18. Stoffel, W., M.E. De Tomás and H.G. Schiefer, Ibid. 348:882 (1967).
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RESULTS AND DISCUSSION

During thin layer chromatography of the unsaponifiable fraction, a major band corresponding to cholesterol was noticed. Unlike the

rat skin (3), no visible bands corresponding to 4-methyl sterols and lanosterol could be seen. However the area of the silica gel corresponding to these fractions was scraped into vials and eluted with chloroform. These extracts were saved for gas liquid chromatography. Cholestanol, if present, would overlap with cholesterol in ordinary Silica Gel G chromatography. Hence they were separated by chromatography on 20% $AgNO_3$-impregnated Silica Gel G (Fig. 1). A definite band corresponding to cholestanol is apparent. The bands were eluted and subjected to gas liquid chromatography after trimethyl silylation (4,7). The band corresponding to cholesterol on gas liquid chromatography gave a large peak corresponding to cholesterol and two small peaks corresponding to campesterol and β-sitosterol (retention time relative to cholesterol–3.10 and 3.98, respectively.). The proportion of campesterol was always higher than β-sitosterol, which was consistent with our observation on the plasma and liver of these pigeons (5). No plant sterols were identified in previous studies with rat skin (3). Cholesterol constituted 94.12% of the total sterols, and plant sterols less than 0.2% (Table I). A small peak with a retention time of 2.07 (relative to 5α-cholestane) could not be identified. The band corresponding to cholestanol on gas liquid chromatography gave a single peak corresponding to authentic cholestanol. On quantitation it amounted to 4.6% of the total sterols. This amount of cholestanol is significant, since the presence of this stanol could not be confirmed in other studies on mammalian skin. The eluates from the area of gel corresponding to 4-methyl sterols and lanosterol gave small peaks, which are tentatively identified (Table I).

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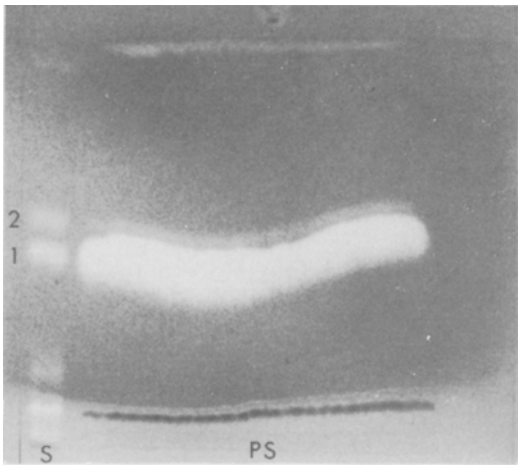

FIG. 1. Thin layer chromatographic separation of skin sterols on $AgNO_3$-impregnated Silica Gel G. S = standards, cholesterol (1) and cholestanol (2). PS = Pigeon skin sterols. Solvent system, chloroform-methanol-acetic acid, 100:1:0.2 v/v. The plates were stained with 2,7-dichlorofluoroscein spray.

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REFERENCES

1. Miller, W.L., Jr., and C.A. Baumann, Proc. Soc. Exp. Biol. Med. 85:561 (1954).
2. Clayton, R.B., A.N. Nelson and I.D. Frantz, Sr., J. Lipid Res. 4:166 (1963).
3. Horlick, L., and J. Avigan, Ibid. 4:160 (1963).
4. Subbiah, M.T.R., B.A. Kottke and P.E. Zollman, Comp. Biochem. Physiol. 41:695 (1972).
5. Subbiah, M.T.R., B.A. Kottke and I.A. Carlo,

TABLE I

Identification and Composition of Pigeon Skin Sterols

Sterol	Thin layer chromatographic behavior[a]	Retention times on gas liquid chromatography[b]	% Composition[c]
Cholesterol	Δ^5-Sterol	2.40	94.2
Cholestanol	5α-Stanol	2.39	4.6
Campesterol	Δ^5-Sterol	3.10	0.2
β-Sitosterol	Δ^5-Sterol	3.98	
Unknown	Monoene sterol	2.07	<1.0
4α-Methyl-5α-cholest-7-en-ol[d]	4-Methyl sterol	3.71	Trace
Lanost-8-en-3β-ol[d]	30-Carbon sterol	3.78	Trace

[a]Based on migration in Silica Gel G and $AgNO_3$-impregnated Silica Gel G chromatography.

[b]Retention times relative to 5α-cholestane.

[c]Absolute sterol concentration 4.2-4.35 mg/g wet tissue.

[d]Tentative identification only.

Biochim. Biophys. Acta 249:643 (1971).
6. Folch, J., M. Lees and G.H. Sloane Stanley, J. Biol. Chem. 226:497 (1957).
7. Subbiah, M.T.R., B.A. Kottke and I.A. Carlo, Lipids 6:517 (1971).

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Tumor Lipids: Comparison of Glyceryl Ethers of Mouse and Insect Tumors

ABSTRACT

Examination of the lipids of two tumor-bearing mutant strains of fruit fly reveal that they do not have the elevated content of glyceryl ethers typical of many neoplasms of higher animals. Glyceryl ether diesters were absent, and the amounts of alkyl and alk-1-enyl glyceryl ethers of tumorous and normal tissues were similar.

INTRODUCTION

The content and metabolism of lipids in abnormal tissues have received considerable attention with the discovery of large amounts of unusual lipids in a variety of tumors of higher animals and man. Tumor cells have been shown to contain abnormally high quantities of ether-bonded lipids, primarily as alkyl glyceryl ethers, that appeared in the neutral glycerolipids as glyceryl ether diesters (1-4). Alkyl glyceryl ethers were also found at above normal concentrations in phosphoglycerides in many neoplasms obtained from human tissues (5). Alterations in the activities of one or more enzymes regulating the amounts of various ether-lipid precursors appear to account for the increased glyceryl ethers of some tumors (6,7).

Many papers have been published that describe a variety of abnormal inclusions, growths, lesions and other tumor-like structures in insects. Such pseudotumors are generally acknowledged to be non-neoplastic in the true sense of vertebrate neoplasms (8), and only recently has a neoplasm been isolated from insects that bears a resemblance to true tumors (9). The present experiments were done to compare the glyceryl ether content of these two types of insect tumor with each other and with two mouse neoplasms known to contain elevated glyceryl ethers (4).

EXPERIMENTAL PROCEDURES

The various strains of *Drosophila melanogaster* Meign. used in these experiments were maintained on standard cornmeal medium at 25 ± 1 C. Flies from the head tumor strain were examined under the dissecting microscope and were separated by sex into nontumorous, tumorous and severely tumorous groups (predominately males with greater than 90% of the head abnormal). Separated heads and abdomens were retained for analysis of lipids. Larvae from the neuroblastoma strain were dissected and examined microscopically to obtain tumorous and normal tissues. Adults of this strain are all nontumorous. Whole insects or dissected tissues were transferred immediately into chloroform-methanol 2:1 v/v and homogenized. Near terminal mouse mammary adenocarcinoma (BW10232) was dissected from C57BL/6J mice maintained in our laboratory. Ehrlich ascites carcinoma lipids were obtained from Swiss mice. Total lipids were extracted by the Folch technique (10), except for the Ehrlich ascites carcinoma lipids which were recovered by the technique of Bligh and Dyer (11).

The ether-bonded lipids of these extracts were analyzed by thin layer chromatography (TLC) and quantitative photodensitometry as described by Wood and Snyder (4,12). All TLC plates were sprayed with concentrated H_2SO_4 and were charred under uniform conditions for 30 min at 180 C. Known amounts of a standard glyceryl ether (glyceryl-1-octadecyl ether, Analabs, Inc., North Haven, Conn.) were analyzed in triplicate on each plate along with experimental lipids. The standard was purified to >99% purity by preparative TLC before use.

RESULTS AND DISCUSSION

Analyses were made of the glyceryl ether diesters (GEDE), alkyl glyceryl ethers, and alk-1-enyl glyceryl ethers of two mutant tumorous strains, and of a normal nontumorous strain of the fruit fly. The tumorous head mutant, tu^h is generally acknowledged to be a pseudotumor or tumefactive lesion of the adult fly that does not have the same biological

Biochim. Biophys. Acta 249:643 (1971).
6. Folch, J., M. Lees and G.H. Sloane Stanley, J. Biol. Chem. 226:497 (1957).
7. Subbiah, M.T.R., B.A. Kottke and I.A. Carlo, Lipids 6:517 (1971).

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Tumor Lipids: Comparison of Glyceryl Ethers of Mouse and Insect Tumors

ABSTRACT

Examination of the lipids of two tumor-bearing mutant strains of fruit fly reveal that they do not have the elevated content of glyceryl ethers typical of many neoplasms of higher animals. Glyceryl ether diesters were absent, and the amounts of alkyl and alk-1-enyl glyceryl ethers of tumorous and normal tissues were similar.

INTRODUCTION

The content and metabolism of lipids in abnormal tissues have received considerable attention with the discovery of large amounts of unusual lipids in a variety of tumors of higher animals and man. Tumor cells have been shown to contain abnormally high quantities of ether-bonded lipids, primarily as alkyl glyceryl ethers, that appeared in the neutral glycerolipids as glyceryl ether diesters (1-4). Alkyl glyceryl ethers were also found at above normal concentrations in phosphoglycerides in many neoplasms obtained from human tissues (5). Alterations in the activities of one or more enzymes regulating the amounts of various ether-lipid precursors appear to account for the increased glyceryl ethers of some tumors (6,7).

Many papers have been published that describe a variety of abnormal inclusions, growths, lesions and other tumor-like structures in insects. Such pseudotumors are generally acknowledged to be non-neoplastic in the true sense of vertebrate neoplasms (8), and only recently has a neoplasm been isolated from insects that bears a resemblance to true tumors (9). The present experiments were done to compare the glyceryl ether content of these two types of insect tumor with each other and with two mouse neoplasms known to contain elevated glyceryl ethers (4).

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Analyses were made of the glyceryl ether diesters (GEDE), alkyl glyceryl ethers, and alk-1-enyl glyceryl ethers of two mutant tumorous strains, and of a normal nontumorous strain of the fruit fly. The tumorous head mutant, tu^h is generally acknowledged to be a pseudotumor or tumefactive lesion of the adult fly that does not have the same biological

TABLE I

Content of Glyceryl Ethers in Tumorous-Head (tu^h) and Giant Larva Neuroblastoma (l [2] gl^4) Mutant Strains of the Fruit Fly, and in Two Mouse Tumors

Lipid source	Tissue condition[a]	Glyceryl ether, %[b]	
		Alkyl	Alk-1-enyl
	tu^h strain		
Males (whole)	NT	2.35	0.66
Males (heads)	NT	2.65	0.38
Males (whole)	T	2.04	0.31
Males (heads)	T	3.45	0.12
Males (isolated heads with severe tumors)	T	1.28	0.10
Males (abdomen from severe tumor heads)	T	0.80	0.05
Females (whole)	NT	1.05	0.62
Females (heads)	NT	2.71	0.19
Females (whole)	T	1.65	0.51
Females (isolated heads with severe tumors)	T	4.12	0.12
	Overall averages, tu^h strain		
	NT	2.19	0.46
	T	2.22	0.20
	$l(2)\ gl^4$ strain		
Males and females (whole)	NT	1.29-2.37	0.27-0.36
Males and females, normal stock	NT	1.59	0.54
Puparia (whole)	NT & T mixed	0.12	0.03
Larvae, normal 3rd instar	NT	0.18	0.06
Larvae, giant	T	0.15	0.09
	Mouse tumors		
Mammary adenocarcinoma (neutral lipids)	T	10.59	1.35
Ehrlich ascites carcinoma	T	8.25	1.74

[a] T = tumorous; NT = nontumorous.

[b] Per cent glyceryl ethers based on weighed samples of total lipids except for mammary adenocarcinoma. These percentages have been corrected by multiplying by a factor of 3 the percentages of the free alkyl and alk-1-enyl glycerols determined by thin layer chromatographic photodensitometry.

characteristics as a true neoplasm of higher animals. Such nontransplantable pseudotumors of insects are not invasive or lethal to their hosts, and the tumorous cells do not change their organ-specific histological characteristics (8). The second mutant, lethal giant larva $l(2)\ gl^4$, is an invasive and transplantable neuroblastoma of the larval brain that remarkably resembles true neoplasms in many ways. In this mutant, the brain lobes are enlarged and deformed, and continue to grow in the enlarged larva. Other larval organs are also abnormal. The imaginal discs grow into amorphous cellular clusters; the lymph glands and gonads are abnormal; the fat body is greatly reduced; and the larva is transparent and swollen with excess hemolymph (9).

The results of our analyses of ether-bonded lipids of the insect and mouse tumors were in sharp contrast with each other. Whereas the mouse tumors possessed easily detectable GEDE by TLC analysis, no evidence could be obtained for the presence of GEDE in any of the insect tumors. Even the invasive neuroblastoma proved negative for GEDE. For example, a sample of 90 enlarged brains and imaginal discs was obtained and their total lipids analyzed by TLC. Free fatty acids, cholesterol, steryl esters and a very large amount of triglyceride were found, but there was nothing evident in the TLC position of GEDE when compared with authentic standard (1, 2 dipalmitoyl-3-octadecenyl ether, Analabs, Inc., North Haven, Conn.).

Quantitative photodensitometric analysis of a large selection of tu^h and $l(2)\ gl^4$ tissues and whole insects further revealed that the alkyl and alk-1-enyl glyceryl ethers were far lower

than in mouse neoplasms (Table I). Although some variation in total corrected per cent glyceryl ether is noted in this table for the various insect samples, tumor-bearing tissues often had much lower glyceryl ethers than comparable structures of nontumorous insects. For example, nontumorous heads of male flies of the tu^h strain contained more than twice as much glyceryl ether as males with severe head tumors. The overall average for tumorous vs. nontumorous adults was very similar for both types of glyceryl ether (Table I). There was a tendency for higher amounts of glyceryl ether in heads than in abdomens of whole adult flies. The glyceryl ether content of Ehrlich ascites tumor in Table I agreed quite closely with that reported by Snyder and Wood (4).

In the *l (2) gl*^{4} strain, nontumorous samples contained as much glyceryl ether as comparable tumorous ones. Adults appear to contain much higher glyceryl ethers than the larvae. Such variations in glyceryl ether content and biosynthesis of different developmental stages of insects have been reported before (13,14) and partially account for the variation and higher amounts of ethers detected in the tu^h adults noted in Table I.

Admittedly our selection of only two insect tumors from the large number available limits our observations. However the amount of tissue obtainable from many of the other *Drosophila* lesions is extremely small, and obtaining larger samples would involve prohibitive amounts of time. We believe that the results are particularly significant because of the absence of elevated glyceryl ethers from the invasive and transplantable *l (2) gl*^{4} strain. Although resembling a true neoplasm in many ways, this neuroblastoma does not appear to possess the abnormal ether-lipid pattern characteristic of many vertebrate neoplasms. We hope to be able to report more fully on the lipids of this neuroblastoma when larger amounts of tissue become available.

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REFERENCES

1. Snyder, F., E.A. Cress and N. Stephens, Lipids 1:381 (1966).
2. Bollinger, J.N., Ibid. 2:143 (1967).
3. Wood, R., and F. Snyder, J. Lipid Res. 8:494 (1967).
4. Snyder, F., and R. Wood, Cancer Res. 28:972 (1968).
5. Snyder, F., and R. Wood, Ibid. 29:251 (1969).
6. Soodsma, J.F., C. Piantadosi and F. Snyder, Ibid. 30:309 (1970).
7. Howard, B.V., H.P. Morris and J.M. Bailey, Ibid. 32:1533 (1972).
8. Harshbarger, J.C., and R.L. Taylor, Ann. Rev. Entomol. 13:159 (1968).
9. Gateff, E., and H.A. Schneiderman, Nat. Cancer Inst. Monogr. No. 31:365 (1969).
10. Folch, J., M. Lees and G.H. Sloane-Stanley, J. Biol. Chem. 226:497 (1957).
11. Bligh, E.G., and W.J. Dyer, Can. J. Biochem. Physiol. 31:911 (1959).
12. Wood, R., and F. Snyder, Lipids 3:129 (1968).
13. Lambremont, E.N., and R. Wood, Ibid. 3:503 (1968).
14. Lambremont, E.N., J. Insect Physiol. 18:581 (1972); Comp. Biochem. Physiol. 41B:337 (1972); Lipids 7:528 (1972).

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Activity of Fatty Acyl CoA-Lysophospholipid Acyltransferases in Liver Microsomes of Rats Fed a Choline-Deficient Diet

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ABSTRACT

Feeding a choline-deficient diet to rats has no effect on the activity of fatty acyl CoA-lysophospholipid acyltransferases, even though liver microsomes are severely depleted of lecithins. Thus lecithins appear to have no functional role in the activity of these transferases. It can be excluded that the latter enzymes are involved in the production of the changes in the fatty acid composition of liver phospholipids occurring in choline-deficient rats. These changes result most likely from alterations in the biosynthesis of liver lecithins and cephalins.

INTRODUCTION

Feeding a choline-deficient (CD) diet to rats leads to changes in the fatty acid composition of lecithins of total liver (1) and of liver microsomes (2) within 24 hr. The changes include a decrease in the percentage of oleic and linoleic acids and a marked increase in the percentage of arachidonic acid. Minor changes are also seen in the fatty acid composition of cephalins (2). (The term "cephalins" is used here to indicate phosphatidyl ethanolamines, phosphatidyl monomethylethanolamines and phosphatidyl dimethylethanolamines.) In an attempt to reveal the mechanism responsible for these changes we assayed the activity of fatty acyl CoA-lysophospholipid acyltransferases in microsomes isolated from the liver of CD and choline-supplemented (CS) rats.

Fatty acyl CoA-lysophospholipid acyltransferases are constitutive enzymes of microsomes and afford the cell a mean of retailoring the fatty acid composition of phospholipids (3). Indeed, it has been shown that most of the polyunsaturated fatty acids, such as arachidonic acid, are introduced into liver lecithins by the activity of these acyltransferases after synthesis de novo of the lecithins (4). Feeding a CD diet to rats induces acute changes in the ultrastructure (5) and in the lipid composition of liver microsomes (2). Furthermore phospholipids are known to be essential for the functional integrity of certain enzymes located in membranes (6). For these reasons it was decided to investigate the activity of fatty acyl CoA-lysophospholipid acyltransferases and determine whether an altered activity is a factor involved in producing the changes in fatty acid composition of liver lecithins and cephalins of CD rats.

MATERIALS AND METHODS

General Procedures

Male rats of the Sprague-Dawley strain (Sprague-Dawley Co., Madison, Wis.) weighing ca. 100 g were used. Preparation of animals for the experiments and preparations of the CS and CD diets were performed as previously reported (7). Groups of rats were fed the CS diet

TABLE I

Fatty Acyl CoA-Lysophospholipid Acyltranserase Activities of Rat Liver Microsomes[a]

	Phospholipid added			
	1-Acyl-glycerophosphoryl choline		1-Acyl-glycerophosphoryl ethanolamine	
Acyl CoA added	Choline-supplemented	Choline-deficient	Choline-supplemented	Choline-deficient
16:0	4.9 ± 0.9	4.4 ± 0.8	1.4 ± 0.5	1.5 ± 0.2
18:0	4.4 ± 0.4	3.6 ± 0.4	2.1 ± 0.3	1.7 ± 0.4
18:1	24.2 ± 1.3	25.0 ± 1.6	4.4 ± 0.4	3.5 ± 0.8
18:2	36.6 ± 3.2	40.5 ± 2.4	6.7 ± 0.6	7.3 ± 0.3
20:4	45.8 ± 1.2[b]	50.5 ± 1.4[b]	10.0 ± 1.1	9.8 ± 0.7
22:6	7.9 ± 0.9	9.1 ± 1.1	1.0 ± 0.3[b]	1.4 ± 0.3[b]

[a]Each value represents nmol/min/mg microsomal protein and is the mean ± SEM obtained from four rats, except those indicated. Liver microsomes were isolated from rats fed one of the two diets for 24 hr.

[b]Mean ± SEM obtained from eight rats.

(control animals) or the CD diet (experimental animals) for 24 hr. The animals had access to the diets up to the time of sacrifice.

Assay of Fatty Acyl CoA-Lysophospholipid Acyltransferases

The liver was homogenized in 3 volumes of 0.25 M sucrose to 0.002 M EDTA, pH 7.4, with a Polytron (Model PT 10, Brinkman Inst. Inc., Westbury, N.Y.) at the lowest speed for 45 sec. The homogenate was centrifuged at 20,000 x g for 20 min in a Spinco Model L centrifuge. The supernatant was collected and centrifuged at 105,000 x g for 60 min to yield microsomal pellets. The pellets were washed once by resuspension in the sucrose-EDTA medium and by recentrifugation at 105,000 x g for 60 min. The final pellets were suspended in sucrose-EDTA medium to a concentration of ca. 10 mg microsomal protein per milliliter. The suspensions were stored at -15 C (8). Microsomal proteins were determined by the method of Lowry et al. (9).

Coenzyme A thioesters of oleic, linoleic, arachidonic and docosahexaenoic acids were prepared from CoA (lithium salt, Nutritional Biochemicals Corp., Cleveland, Ohio) and fatty acids (Analabs, Inc., North Haven, Conn.) as described by Goldman and Vagelos (10). Yields varied from 15 to 50% based on comparing the content of the sulfhydryl group of the starting material and the extent of CoA released in the assay system. About 80% of each fatty acyl CoA was found to be active in the assay system. This assessment was based on comparing the phosphorus content of the fatty acyl CoA preparations and the extent of CoA released in the essay system (8,11). S-Palmitoyl CoA and S-stearoyl CoA were purchased from Sigma Chemical Co. (St. Louis, Mo.). The thioesters were dissolved in distilled water, saturated with Santoquin (Monsanto, St. Louis, Mo.), to a concentration of 2×10^{-3} M and stored at -15 C (11). 1-Acyl-glycerophosphoryl choline was obtained from General Biochemicals (Chagrin Falls, Ohio), and 1-acyl-glycerophosphoryl ethanolamine from Supelco Co. (Bellefonte, Pa.). The lysophospholipids were dispersed in water (8) with the aid of a Polytron operated at maximal speed for 5 min.

Acyltransferase activities were assayed as indicated by Lands et al. (8). The sulfhydryl group binding reagent was prepared by dissolving 39.6 mg of 5,5′-dithiobis (2-nitro-benzoic acid) (DNTB, Aldrich Chemical Co., Milwaukee, Wis.) in 10 ml of 0.1 M phosphate buffer, pH 7.0 (12). The complete assay mixture contained, in a final volume of 1.0 ml: 0.7 ml of 0.1 M Tris-HCl buffer, pH 7.4; 0.1 ml of 0.01 M DNTB; 25 to 30 nmol of fatty acyl CoA; 0.15 to 0.20 mg of microsomal proteins, and 0.1 ml of 1-acyl-lysophospholipid (180 nmol of 1-acyl-GPC, or 150 nmol of 1-acyl-GPE). Rates of CoA release were determined in complete mixtures and in mixtures without 1-acyl-lysophospholipid, and the rate of acyl transfer was calculated from the difference of the two. The increase in absorbance at 412 mμ was measured in a Cary Model 16 spectrophotometer. Duplicate analyses were performed on the microsomal preparation of each rat.

Differences between the means were checked with student t test and regarded as significant if $P \leqslant 0.05$.

RESULTS

The results are presented in Table I. No significant difference was seen in the activity of acyltransferases between CS and CD rats.

As observed by Lands et al. (11,13), the rate of acyl transfer from the thioesters of unsaturated fatty acids is higher than that from the esters of saturated fatty acids when 1-acyl-lysophospholipid is used as an acceptor; for each fatty acyl CoA acylation of 1-acyl-glycerophosphoryl choline (GPC) is considerably faster than that of 1-acyl-glycerophosphoryl ethanolamine (GPE). Among the six fatty acyl CoA's tested, arachidonic acyl CoA exhibits the highest rate of acylation. This is in agreement with the findings of Holub et al. (4), that a large portion of arachidonic acid-containing lecithins in liver are derived from acyltransfer.

DISCUSSION

Fatty acyl CoA-lysophospholipid acyltransferases are localized in the microsomal fraction of liver homogenates (3). Choline deficiency induces acutely marked alterations in the ultrastructural appearance of the endoplasmic reticulum of hepatocytes, as well as a severe depletion of lecithins in the microsomal membranes (2,5). However the results of this study indicate that these alterations have no repercussion on the activity of the acyltransferases. Therefore the functional role of lecithins in the activity of acyltransferases appears limited. This conclusion corroborates the observation by Ellingson et al. (3) that partial removal of microsomal phosphoglycerides by treatment with phospholipases failed to demonstrate a functional requirement for diacylphosphoglycerides in acyltransferase activity.

As pointed out recently (4), at least three different biosynthetic pathways are of importance in determining the fatty acid composition

of liver lecithins: synthesis via phosphatidic acid and CDP-choline (14); stepwise methylation of phosphatidyl ethanolamines to lecithins (15), and the acylation of lysolecithins (3). Oleic and linoleic acid-containing lecithins derive mostly from the synthesis via phosphatidic acid and CDP-choline, with acylation of lysolecithins playing a less important role (4). On the other hand, arachidonic acid-containing lecithins appear to originate mostly from acylation of lysolecithins, with the stepwise methylation of phosphatidyl ethanolamines being of secondary importance (4).

Thus, to the extent that results obtained in vitro can be extrapolated to situations in vivo, fatty acyl CoA-lysophospholipid acyltransferases appear to play no direct role in producing the changes in the fatty acid composition of liver phospholipids observed in rats fed a CD diet. In a previous study (1), evidence was obtained suggesting that the methylation of phosphatidyl ethanolamines to lecithins is increased in the liver of CD rats. Lecithins derived from methylation of phosphatidyl ethanolamines have been demonstrated to be particularly rich in arachidonic acid (16,17). Therefore the changes induced by choline deficiency in the fatty acid composition of liver lecithins, which show a marked increase in the percentage of arachidonic acid, are most likely the result of alterations in the synthesis de novo of liver lecithins.

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REFERENCES

1. Lombardi, B., P. Pani, F.F. Schlunk and S.-H. Chen, Lipids 4:67 (1969).
2. Chen, S.-H., L.W. Estes and B. Lombardi, Mol. Exp. Pathol. 17:176 (1972).
3. Ellingson, J.S., E.E. Hill and W.E.M. Lands, Biochim. Biosphys. Acta 196:176 (1970).
4. Holub, B.J., W.C. Breckenridge and A. Kuksis, Lipids 6:307 (1971).
5. Estes, L.W., and B. Lombardi, Lab. Invest. 21:374 (1969).
6. van Deenen, L.L.M., and G.H. Haas, Ann. Rev. Biochem. 35:157 (1966).
7. Lombardi, B., G. Ugazio and A.N. Raick, Amer. J. Physiol. 210:31 (1966).
8. Lands, W.E.M., and P. Hart, J. Biol. Chem., 240:1905 (1965).
9. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, Ibid. 193:265 (1951).
10. Goldman, P., and P.R. Vagelos, Ibid. 236:2620 (1961).
11. Lands, W.E.M., M.L. Blank, L.J. Nutter and O.S. Privett, Lipids 1:224 (1966).
12. Ellman, G.L., Arch. Biochem. Biophys. 82:70 (1959).
13. Lands, W.E.M., and P. Hart, JAOCS 43:290 (1966).
14. Kennedy, E.P., Fed. Proc. 21:934 (1961).
15. Astom, C., and H.B. Lofland, Biochem. Biophys. Res. Com. 3:244 (1960).
16. Isozaki, M., A. Yamamoto, T. Amako, Y. Sakai and H. Okita, Med. J. Osaka Univ. 126:285 (1962).
17. Lyman, R.L., J. Tinoco, P. Bouchard, G. Sheehan, R. Ostwald and P. Miljanich, Biochem. Biophys. Acta 127:107 (1967).

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Studies on Detection and Synthesis of Prostaglandins in Tail Skin of the Rat

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ABSTRACT

Studies are reported on the detection and in vitro biosynthesis of prostaglandins of the E type in the tail skin of male Sprague-Dawley rats. It was found that strips of skin were preferred to other techniques of skin preparations, such as mincing or homogenization for the assay of endogenous prostaglandins. Each strip of tail skin could be used at least twice, each time in fresh buffer to obtain additional release of endogenous prostaglandins. The skin was stripped from a longitudinal incision in the tail in one piece and cut into four parallel strips lengthwise. Each strip of skin was incubated singly and the prostaglandins were extracted with ethyl acetate. The detection and analysis of prostaglandins of the E type were carried out by standard procedures on the residue of this extract dissolved in 95% ethanol. By means of this technique, endogenous prostaglandins were detected in the tail skin of rats. Little or no prostaglandins were extracted by organic solvents directly from the tissue, and the yields were low when the tissue was minced. The results indicate the possibility that endogenous prostaglandins may be synthesized during incubation in buffer or may be released from binding with other cellular components. The major prostaglandin of this tissue was PGE_2; prostaglandin E_1 (PGE_1) was also detected. Using the same procedure, the presence of a prostaglandin synthetase system in the tail skin was demonstrated by the conversion of exogenous arachidonic acid to prostaglandin E_2 (PGE_2).

INTRODUCTION

Recently (1-3), the occurrence of scaliness in the tail skin of hypophysectomized rats similar to that in essential fatty acid deficiency has been demonstrated. Scaliness of tail has been attributed to a deficiency of prostaglandins (4) which are found to be actively synthesized in the skin of rat (5).

In general, the assay of prostaglandins in skin and in other tissue is carried out on homogenates (5-8). In a previous communication (9), we showed that grinding the tissue in the frozen state was preferred to homogenization for the assay of prostaglandins in rat vesicular glands. In the present study a simple technique was developed for the assay of prostaglandins in tail skin which is suitable for application to EFA-deficient and hypophysectomized animals. The technique is based on the use of strips of skin in preference to homogenization or mincing the tissue.

MATERIALS AND METHODS

Animals

Normal male rats of the Sprague-Dawley strain, 180-200 g body wt, were obtained from the Hormone Assay Lab., Chicago. The animals were housed in individual cages and fed ad libitum a basic fat-free diet supplemented with vitamins and minerals in the required amounts (1,3) and 10% corn oil.

TABLE I

Detection of Endogenous PGE in Tail Skin of Male Rats

Experiment no.	1st Incubation,[a] μg/g	2nd Incubation,[a] μg/g	PGE, μg/g
1	57	42	99
2	73	47	120
3	51	28	79
4	122	34	156
5	47	41	88

[a]The same strip of skin from each rat tail was incubated twice, each time in new buffer for 1 hr. 10 ml buffer was used per g of skin from tail. Calculations were based on μg of PGE released per gram of tail skin. Other experimental conditions are described in the text.

TABLE II

Effect of pH on PGE Release from Rat Tail Skin[a]

pH	PGE release, μg/g
4	76 ± 7[a]
7	89 ± 8
8.5	115 ± 11
9.2	132 ± 6

[a]M ± SD, three animals. Conditions: 20 ml of 0.1 M NH_4Cl/g tail skin; pH adjusted with concentrate NH_4OH or HCl.

TABLE III

Effect of Buffer Volume on PGE Release from Rat Tail Skin after Incubation

ml Buffer/g tail skin	μg PGE/g tail skin
5	48 ± 5[a]
10	70 ± 22
20	115 ± 11

[a]M ± SD, three animals. Conditions: 0.1 M NH_4Cl; pH 8.5, 2 mg GSH/ml buffer; incubation time 1 hr.

Preparation of Skin from Tail

The rats were sacrificed under ether. The tails were cut and placed on dry ice when not immediately used and stored at -20 C. For each assay a lengthwise incision was made in the tail; the skin was stripped in one piece and cut lengthwise into four parallel strips. Each strip of skin was incubated individually in a 25 ml beaker. Usually a 200 g rat yielded ca. 2 g of tail skin.

Incubation and Extraction

Strips of skin of ca. 0.7 g each were incubated in 10 ml of 0.1 M NH_4Cl buffer containing 20 mg glutathione at final pH 8.5. For enzymic conversion of arachidonic acid to prostaglandins, concentrations of arachidonic acid from 1 to 6 mg/g skin were used. The solvent was evaporated under nitrogen from a solution of the substrate which was placed in 25 ml beaker and emulsified with buffer. A strip of skin was then immersed in the emulsion and incubated in an atmosphere of air for 1 hr at 37 C with shaking. Systems without the substrate or without skin were used as controls. The reaction was stopped by adjusting the pH of the incubation mixture to pH 3 with 1 M citric acid. After removing the skin strip, the reaction mixture was extracted three times with 40 ml ethyl acetate. The ethyl acetate of the combined extracts was evaporated under negative pressure and the products were dissolved in 5 ml 95% ethanol. Aliquots of the ethanol solution were used for identification and quantitative determination of prostaglandins.

Assay

Prostaglandin reference standards (95% pure) and arachidonic acid (99% pure) were obtained from the Lipids Preparation Lab. of The Hormel Institute. The absorption by PGE_2 at 278 mμ after alkali addition was plotted as a function of time, and the optical density at zero time was determined by extrapolation (10,11). The total amount of PGE_2 was calculated on the basis of the known molar extinction coefficient. (ΔO.D. = 1.00 corresponds to 39.4 μg PGE compounds per 3 ml reaction mixture [10,11].)

Thin Layer Chromatography (TLC)

The TLC techniques used for the identification and isolated of the prostaglandins and the subsequent resolution of PGE_1, PGE_2 and PGE_3 by $AgNO_3$/TLC have been described by Green and Samuelsson (12) and Ramwell and Daniels (13).

RESULTS

Application of the procedure described

TABLE IV

Detection of PGE from Rat Tail Skin in Different Systems

No. of animals	Buffer system	1st Incubation, μg/g	2nd Incubation, μg/g
3	0.2 M Phosphate	44 ± 3[a]	39 ± 4[a]
3	0.2 M Phosphate, plus 6 mg Arachidonic acid/g skin	104 ± 10	79 ± 5
5	0.1 M NH_4Cl	70 ± 22	38 ± 6
4	0.1 M NH_4Cl, using minced tail skin	36 ± 5	
5	0.1 M NH_4Cl, plus 6 mg Arachidonic acid/g skin	126 ± 17	70 ± 10
4	0.1 M NH_4Cl, without Glutathione	15.3 ± 3	19.6 ± 2

[a]M ± SD. Unless otherwise specified, strips of tail skin and glutathione (2 mg/ml) 10 ml buffer pH 8.5 was used per g skin; other conditions described in the text.

TABLE V

Conversion of Arachidonic Acid to PGE_2 by Repeated Use of Same Strips of Rat Tail Skin in Two Successive Incubations[a]

Substrate concentration, mg/g	1st Incubation		2nd Incubation	
	Net synthesis, μg/g	Yield, %	Net synthesis, μg/g	Yield, %
1	15 ± 2	1.5	19 ± 9	1.9
3	33 ± 3	1.1	31 ± 7	1
6	70 ± 10	1.2	41 ± 14	0.7

[a]M ± SD from three rats. Conditions: 0.1 M NH_4Cl, pH 8.5, 10 ml/g tail skin. The same strips of tail skin were used twice, in the first and second incubations, each time with new buffer and substrate. Net synthesis was calculated as: incubation with substrate minus incubation without substrate. All other conditions are described in text.

above to the detection of prostaglandins of endogenous origin in the tail skin of normal rats is shown in Table I. In these experiments, two successive incubations were carried out on the same strip with fresh buffer. Sometimes a third incubation yielded additional prostaglandin. In contrast, no prostaglandins were detected by direct extraction of tail skin with acetone, ethyl acetate or chloroform, although these solvents yielded the fat, fatty acids and other substances from the tail skin.

The effects of pH and volume of buffer on the yield of prostaglandin are shown in Tables II and III, respectively. The optimum volume for the incubation of a skin strip was ca. 20 ml/g of skin and the highest yield of prostaglandin was obtained at alkaline pH. Silver nitrate-TLC of the extracts showed that PGE_2 was the most abundant species of the endogenous prostaglandin fraction. Sometimes trace amounts of PGA and PGB were detected on the TLC plates. However these compounds could have been produced by dehydration during chromatography (5).

Studies of the effect of several other conditions on the yield of prostaglandin are shown in Table IV. These results showed that phosphate buffer could be used in place of ammonium chloride buffer, but the yield of prostaglandin was low. The yield of prostaglandin was low when minced tissue was used, and this technique precluded a second incubation. When glutathione was left out of the incubation mixture, the yield of prostaglandin was also low. The addition of arachidonic acid to the incubation mixture increased the yield of prostaglandin E_2 indicating in vitro biosynthesis of PGE_2. The biosynthesis of PGE_2 from exogenous arachidonic acid is demonstrated in Table V. These results indicated that ca. 1% of the added arachidonic acid substrate was converted to prostaglandins. The fact that a similar yield was obtained in the second incubation showed that the synthetase system was still active and fairly stable during the incubation.

DISCUSSION

The technique described here permits the detection of prostaglandins of endogenous origin in the tail skin of rats. There is some question of whether the prostaglandins are present in the tissue as such, or are synthesized during the procedure inasmuch as the optimum conditions for extraction are similar to those for in vitro biosynthesis. It has been generally observed (6) that homogenization in buffer gave a higher yield of prostaglandin than homogenization in alcohol. These observations have been interpreted to indicate endogenous synthesis during preparation of the tissue. Conceivably, endogenous synthesis of prostaglandins could occur during the incubation procedure prior to extraction as a result of the liberation of precursor fatty acids by the action of lipases in accordance with the hypothesis of a regulatory function of these enzymes (14,15). This explanation appears inviting because the release of prostaglandins is increased by the presence of glutathione which is believed to facilitate the formation of prostaglandins of the E structures (16).

An alternative to the above explanation is that the incubation-extraction procedure is more efficient than other direct extraction methods with organic solvents to obtain the release of endogenous prostaglandins which may presumably be bound, at least in part, to cellular membranes (17). Little is known yet of the mechanism of prostaglandin's release from the membrane site. However the fact that prostaglandin release is increased by incubation in a buffered solution prior to extraction, by the alkaline pH and by the optimum incubation volume, would seem to indicate the possibility of bound prostaglandins. Another evidence of

endogenous bound prostaglandins comes from the observation of tail skin of hypophysectomized rats, which has lost the ability to synthesize prostaglandins from added precursors yet still has endogenous release of prostaglandins when subjected to similar incubation and extraction procedure (2).

The presence of an active prostaglandin synthetase system in the tail skin of the rat was well demonstrated by the in vitro synthesis of PGE_2 from exogenous arachidonic acid. Studies on whole skin of the rat by van Dorp (6) indicate that the major site of prostaglandin synthesis in skin is in the epidermis. Although only two successive incubations were carried out on each skin strip in the present study, the recoveries were comparable to that obtained by van Dorp on whole skin.

ACKNOWLEDGMENTS

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REFERENCES

1. Haeffner, E.W., and O.S. Privett, J. Nutr., In press.
2. Tan, W.C., and O.S. Privett, manuscript in preparation.
3. Privett, O.S., F. Phillips, T. Fukazawa, C.C. Kaltentach and H. Sprecher, Biochim. Biophys. Acta 280:348 (1972).
4. Ziboh, V.A., and S.L. Hsia, J. Lipid Res. 13:458 (1972).
5. Ziboh, V.A., and S.L. Hsia, Arch. Biochem. Biophys. 146:100 (1971).
6. van Drop, D.A., Ann. N.Y. Acad. Sci. 180:181 (1970).
7. Anggard, E., Ibid. 180:200 (1970).
8. Shaw, J.E., and P.W. Ramwell, Methods Biochem. Anal. 17:325 (1969).
9. Tan, W.C., and O.S. Privett, Lipids 7:622 (1972).
10. Nugteren, D.H., R.K. Beerthuis and D.A. van Dorp, Recueil 85:405 (1966).
11. Yoshimoto, A., H. Ito and K. Tomita, J. Biochem. 68:487 (1970).
12. Green, K., and B. Samuelsson, J. Lipid Res. 5:117 (1964).
13. Ramwell, P.W., and E.G. Daniels, in "Chromatography of the Prostaglandins," Vol. II, Edited by C.V. Marinetti, Marcel Dekker, New York, 1968.
14. Kunze, H., and W. Vogt, Ann. N.Y. Acad. Sci. 180:123 (1971).
15. Lands, W.E., and B. Samuelsson, Biochim. Biophys. Acta 164:426 (1968).
16. Lands, W., R. Lee and W. Smith, Ann. N.Y. Acad. Sci. 180:123 (1971).
17. Piper, P., and J. Vane, Ibid. 180:363 (1971).

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Lipid Metabolism Following Triglyceride Ingestion in Intact and Adrenalectomized Rats

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ABSTRACT

Although both adrenalectomized and intact Long Evans rats absorbed the same amount of fat over a 24 hr period, the livers of the intact rats accumulated triglyceride while the livers of the adrenalectomized rats failed to do so. Of the glycerides excreted in the feces, the adrenalectomized rat excreted a significantly higher proportion of triglycerides while the intact rats excreted primarily monoglycerides. Fecal excretion of bile acids and phospholipids was significantly lower in the adrenalectomized rats compared to intact rats. Thus adrenal hormones appear to influence intestinal lipolysis. Serum and liver phospholipids appeared to be significantly lower in response to a fat load in adrenalectomized rats compared to intact rats. The phospholipid content of the small intestinal wall was five-fold higher in the intact rats than in the adrenalectomized rats. The adrenal gland appears to control phospholipid synthesis during triglyceride assimilation.

INTRODUCTION

Shafrir et al. (1) and Salvador et al. (2) reported that epinephrine caused the mobilization of plasma free fatty acids, while Barrett (3) noted that changes in lipoproteins could be caused by increases in adrenocortical hormone secretion. Preliminary experiments (4) indicated differences in metabolic response of intact and adrenalectomized rats to triglyceride ingestion. Seven hours after triglyceride ingestion there was a 64% rise in plasma glucose, $P < 0.05$, compared to the zero time level, while in the adrenalectomized rat a 29% drop was noted in plasma glucose, $P < 0.01$. In the intact rat, this glucose rise was traced to glucocorticoid release and gluconeogenesis from amino acids (5). It was further noted that plasma phospholipids and cholesterol rose dur-

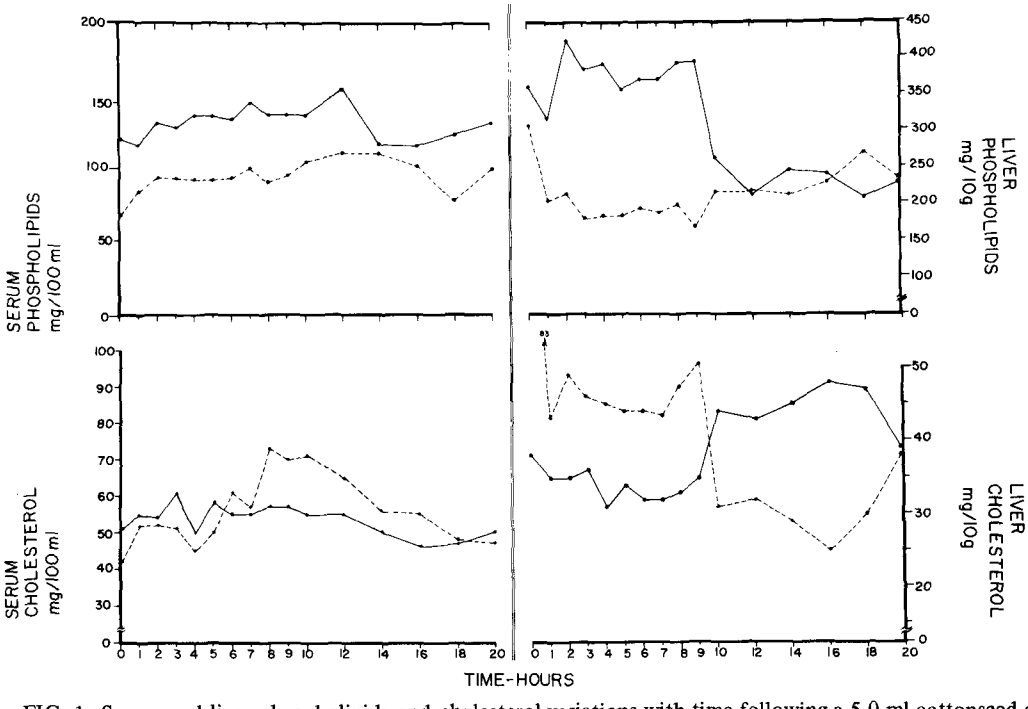

FIG. 1. Serum and liver phospholipids and cholesterol variations with time following a 5.0 ml cottonseed oil load in intact (——) and adrenalectomized (– – –) Long Evans rats.

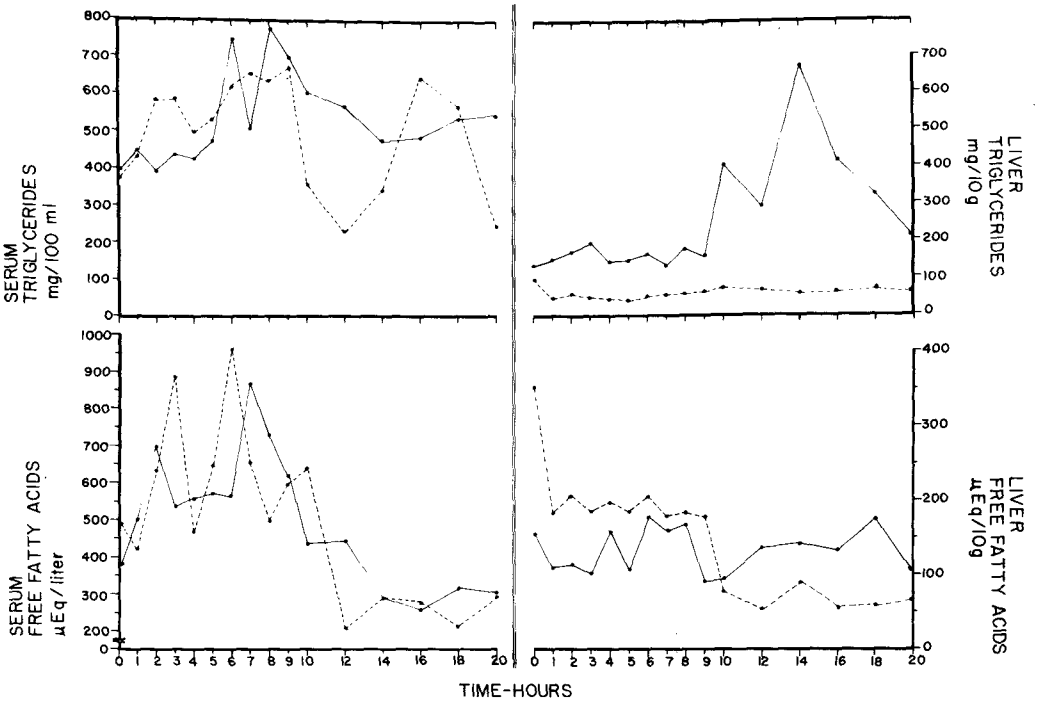

FIG. 2. Serum and liver triglyceride and free fatty acid variation with time following a 5.0 ml cottonseed oil load in intact (——) and adrenalectomized (– – –) Long Evans rats.

ing the first 4 hr after triglyceride loading in intact rats, but not in the adrenalectomized rats.

In an attempt to gain some further understanding of the adrenal control of lipid metabolism, a detailed study of the ways in which intact and adrenalectomized rats respond to triglyceride loading was conducted. The results of this study are presented in this report.

MATERIALS AND METHODS

Sixty-day-old male rats from an inbred colony of the Long Evans strain were used. They were weaned at 23 days of age, and maintained on a commercial rat diet (Purina Lab. Chow) and tap water ad libitum. Three hundred and sixty rats, mean weight 214 ± 4.5 g, were divided into two groups. One group was adrenalectomized by the method described by Ingle and Griffith (6), ensuring that the glands were removed encapsulated; the other group served as intact controls. No replacement therapy was given following adrenalectomy. At the start of the experiment, all animals were 18 hr postprandial; adrenalectomized animals were 5 days postoperative. At the time of oil gavage, the mean weight of the intact rats was 221 ± 5.4 g, while that of the adrenalectomized rats was 206 ± 9.4 g. Five milliliters (4.630 g) of cottonseed oil was given to each rat by gavage. At specific time periods for the next 24 hr, rats were exsanguinated by decapitation. Blood was allowed to clot, and the serum was separated and assayed immediately. Livers were immediately removed, weighed and extracted for assay as previously described (7).

Twenty-four hour fecal collections were extracted for lipids and bile acids as previously described (7). The contents of the stomachs and large and small intestines were removed by washing out with physiological saline solution. The contents were lyophilized to dryness and extracted as previously described for feces (7), except that they were made to a final volume of 10 ml. Small intestinal walls were extracted by grinding with 20 volumes of chloroform-methanol 2:1, washed with isotonic saline, and made to a final volume of 10 ml with chloroform.

Serum phospholipids, cholesterol, triglycerides and free fatty acids were determined by previously described automated methods (8). Liver, stomach contents, intestinal wall, intestinal contents and fecal lipid extracts were assayed by the same procedures. Total lipids were determined gravimetrically.

Fecal mono-, di- and triglycerides were

TABLE I

Fecal Lipids in Intact and Adrenalectomized Rats
24 hr after a 5 ml Triglyceride Load[a]

Lipid	Intact	Adrenalectomized
Total feces, g/day	3.222 ± 0.231[b]	3.681 ± 0.375
Total lipids, mg/day	877 ± 96	905 ± 91
Lipid phosphorus as phospholipids, mg/day	99 ± 13.7	23 ± 10.3[c]
Sterols, 3-β-hydroxy-5-ene, mg/day	15 ± 2.2	17 ± 1.1
Free fatty acids μEq/day	1297 ± 130	1156 ± 99
Bile acids, mg/day	23 ± 2.8	10 ± 2.8[c]
Total glycerides as triolein, mg/day	247 ± 41	431 ± 46[c]
Per cent of total glycerides		
Triglycerides	11.25 ± 2.72	30.35 ± 9.48[d]
Diglycerides	24.50 ± 3.15	37.51 ± 6.42[d]
Monoglycerides	63.96 ± 3.78	32.38 ± 3.39[c]

[a]Ten rats per group.
[b]Mean ± SEM.
[c]$P<0.01$.
[d]$P<0.05$.

separated by column chromatography on silicic acid, employing graded concentrations of diethyl ether in hexane according to the method of Schrade et al. (9).

Analyses for changes were performed on the data by the use of appropriate estimation of error and student *t* tests (10).

RESULTS

The serum lipid responses to a triglyceride load in both intact and adrenalectomized rats are shown in Figures 1 and 2. Serum phospholipids (Fig. 1) were significantly higher in the intact rats than in the adrenalectomized rats throughout the experimental period, $P < 0.05$. Following a triglyceride gavage, in the intact rats a gradual rise in serum phospholipids was noted from the zero time level of 119 ± 5.1 mg/100 ml to 156 ± 12.4 mg/100 ml at 12 hr, $P < 0.01$, followed by a decline to the zero time level by 20 hr. In the adrenalectomized rats, the serum phospholipids rose from a zero time level of 67 ± 5.9 mg/100 ml to 91 ± 3.6 mg/100 ml at 4 hr, $P < 0.05$, and remained elevated throughout the study period.

No significant difference was noted in the serum cholesterol between the intact and adrenalectomized rats at zero time. Following triglyceride ingestion, serum cholesterol in the intact rats did not vary significantly from the zero time level of 51 ± 2.7 mg/100 ml throughout the study period. In the adrenalectomized rats there was no significant variation from the zero time level of 42 ± 3.6 mg/100 ml for the first 4 hr, after which there was an apparent rise (peaking at 175% over the zero time level) through the 8th hr, followed by a gradual decline toward the zero time level by 20 hr. However, because of the large variance, this rise did not attain statistical significance.

In the intact rats, serum triglycerides (Fig. 2) remained at approximately the zero time level of 397 ± 48.1 mg/100 ml until the 4th hr, after which there was a gradual rise to a peak of 773 ± 128.5 mg/100 ml at 8 hr, $P < 0.05$. This rise was followed by a decline toward the zero time level by 14 hr, after which the serum triglycerides remained essentially at the zero time level until the end of the experiment. In the adrenalectomized rats, the peak elevation in serum triglycerides also occurred at ca. 8 hr but was of lesser magnitude (68%, $P < 0.05$) than that of the intact rats (95%, $P < 0.05$), compared to zero hour, after which a sharp decline occurred to the 14th hr, followed by a second rise by the 18th hr.

Serum free fatty acids in the intact rats (Fig. 2) rose from a zero time level of 378 ± 83.0 μEq/L to an initial peak of 694 ± 148.3 μEq/L at 3 hr after triglyceride ingestion. After declining toward the zero time level by 4 hr, they rose to a second peak of 863 ± 281.9 at 7 hr and then declined toward the zero time level by the 12th hr. The decline in serum free fatty acids continued below the zero time level to 258 ± 14.7 μEq/L by the 16th hr, and remained at this lower level until the end of the experimental period. An initial rise in serum free fatty acids from a zero time level of 487 ± 102.4 μEq/L to 884 ± 189.4 μEq/L was noted at 3 hr after triglyceride ingestion in the adrenalecto-

TABLE II

Lipids in Intestinal Lavages of Intact and Adrenalectomized Rats 24 hr after a 5 ml Triglyceride Load[a]

Lipid	Quantity per entire lavages			
	Small intestine and stomach		Large intestine	
	Intact	ADX[b]	Intact	ADX
Total lipids, mg	11.5 ± 0.6[c]	105.3 ± 12.7	101.4 ± 7.7	374.5 ± 50.4[d]
Lipid phosphorus, mg	2.4 ± 0.2	4.0 ± 1.1	4.2 ± 1.4	6.6 ± 1.9
Sterols, 3-β-hydroxy-5-ene, mg	1.3 ± 0.2	3.0 ± 0.8	8.4 ± 0.7	8.3 ± 1.6
Total glycerides, mg	0.8 ± 0.1	3.0 ± 0[d]	2.3 ± 0.1	2.6 ± 0.4
Free fatty acids, μEq	10.5 ± 2.0	345.0 ± 45.0[d]	99.8 ± 8.7	727.5 ± 102.8[d]

[a]Ten rats per group.

[b]Adrenalectomized.

[c]Mean ± SEM.

[d]$P<0.01$.

mized rats, followed by a decline toward the zero time level. A second peak of 958 ± 179.6 μEq/L was observed at 6 hr, followed by a second decline below the zero time concentrations to a level of 208 ± 37.5 μEq/L by 12 hr. The peak free fatty acid concentrations in both the intact and adrenalectomized rats occurred approximately at the same time (6-7 hr); however there were no significant differences throughout the study period in either group due to the high variability between animals.

In the intact rats there was no significant change in liver total lipids (Fig. 3) from the zero time level of 460 ± 51.9 mg/10 g liver for the first 7 hr. Between the 7th and 10th hr there was a rise to 759 ± 58.1 mg/10 g liver, $P < 0.01$, followed by a gradual decrease toward the zero time level. This rise occurred just after the major elevation in serum triglycerides. The rise in liver total lipids to a peak value which was noted in the intact rats was absent in the adrenalectomized rats. There was a 31% decline from the zero level of 509 ± 139.7 mg/10 g liver within 1 hr after the triglyceride load in the adrenalectomized rats. The liver triglycerides remained significantly lower in the adrenalectomized rats than in the intact rats throughout the study period, $P < 0.01$.

Liver phospholipids (Fig. 1) were higher in the intact rats than in the adrenalectomized rats from 2 hr through 9 hr, $P < 0.01$, after which no significant differences were noted between the two groups. In the intact rats there was no significant change from the zero time value of 359 ± 34.9 mg/10 g liver until the 10th hr, after which phospholipids declined to 212 ± 32.1 mg/10 g liver by 12 hr and remained at this lower level to the end of the study period. No statistically significant changes were noted in the liver phospholipids of the adrenalectomized rats throughout the experimental period.

Liver cholesterol (Fig. 1) remained essentially near the zero time level of 38 ± 2.8 mg/10 g liver for the first 9 hr in the intact rats, rose to 48 ± 2.3 mg/10 g liver by 16 hr, $P < 0.05$, and then returned to the zero time level by 20 hr. In the adrenalectomized rats, liver cholesterol decreased from a zero time level of 83 ± 22.2 mg/10 g liver to 25 ± 2.6 mg/10 g liver at 16 hr, $P < 0.05$, after which it returned toward the zero time level.

Liver triglycerides (Fig. 2) in the intact rats remained essentially at the zero time level of 131 ± 25.0 mg/10 g liver for the first 9 hr, then abruptly rose to 673 ± 167.1 mg/10 g liver by the 14th hr, $P < 0.05$, and finally declined toward the zero time level. In the adrenalectomized rats, the liver triglycerides did not significantly change from the zero time level of 97 ± 30 mg/10 g liver throughout the experimental period. Liver triglycerides were lower in the adrenalectomized rats than in the intact rats, $P < 0.01$. The differences noted in liver total lipids of the intact rats were essentially due to the changes in liver triglycerides.

No significant variability was noted in the liver free fatty acids of the intact rats in response to triglyceride loading (Fig. 2). In the adrenalectomized rats, there was an initial decrease during the 1st hr and a further decrease between the 9th and 10th hr. The large variance precluded statistical significance.

Twenty-four hour fecal collections were examined in order to determine whether there was a significant difference in fat excretion (Table I). No significant differences between the two groups were noted in the fecal weight, fecal total lipids, fecal 3-β-hydroxysterols, or total free fatty acids. Total glyceride excretion was almost twice as high in the adrenalectomized rats as in the intact rats, $P < 0.01$. When the glycerides were separated chromatographi-

TABLE III

Lipids in Small Intestinal Walls of Intact and Adrenalectomized Rats after a 5 ml Triglyceride Load[a]

Lipid	Quantity per entire small intestine	
	Intact	Adrenalectomized
Total lipids, mg	428.5 ± 33.24[b]	645.9 ± 76.88[c]
Phospholipids, mg	72.0 ± 9.30	15.8 ± 1.49[d]
Cholesterol, mg	19.6 ± 1.43	18.2 ± 1.48
Total glycerides, mg	283.0 ± 23.2	508.0 ± 56.11[c]
Free fatty acids, μEq	62.3 ± 7.40	179.0 ± 30.20[d]

[a]Ten rats per group.
[b]Mean ± SEM.
[c]$P<0.05$.
[d]$P<0.01$.

cally on silicic acid columns, a significant difference in the degree of hydrolysis was noted between intact and adrenalectomized rats. In the intact rats, 63.96 ± 3.78% of the total fecal glycerides, was in the form of monoglycerides, while in the adrenalectomized rats the percentage of monoglycerides was only 32.38 ± 3.39%, $P < 0.01$. Diglycerides and triglycerides were correspondingly higher in the adrenalectomized than in the intact rats, $P < 0.05$.

Fecal lipid solvent soluble phosphorus excretion was lower in the adrenalectomized rats than in the intact rats, $P < 0.01$ (Table I). Similarly, fecal bile acid excretion in the adrenalectomized rats was only half that found in the intact rats, $P < 0.01$.

In addition to the fecal studies, the contents of both the large and the small intestines were washed out and assayed (Table II). Total lipids, total glycerides and free fatty acids were significantly higher in the small intestinal lavages of the adrenalectomized rats than those of the intact rats, $P < 0.01$. In the large intestinal lavages of the adrenalectomized rats, total lipids and free fatty acids were higher than in the intact rats, $P < 0.01$. Nevertheless the difference in total lipids between the intact and adrenalectomized rats found in the lavages was only ca. 300 mg out of a total of 5 ml (4.630 g) of fat given. The possibility existed that significant amounts of fat remained in the stomach. Analysis of the stomach contents showed no significant differences between intact and adrenalectomized rats; the total recovered lipid was less than 1 mg. Thus, since the gavaged triglycerides could not be recovered in either the gastrointestinal tract or the feces, it would appear that the fat was absorbed.

The small intestinal walls were extracted and assayed for lipids in rats 24 hr postingestion (Table III). Total lipids were ca. 200 mg higher in the small intestinal walls of the adrenalectomized rats than the intact rats, $P < 0.05$. This difference was primarily in the form of glycerides, since intestinal wall glycerides in the adrenalectomized rats were ca. 225 mg higher than in the intact rats, $P < 0.05$. Similarly, free fatty acids were higher in the small intestinal walls of the adrenalectomized rats, $P < 0.01$. Conversely, phospholipids were almost five times higher in the intestinal walls of the intact rats than in the adrenalectomized rats, $P < 0.01$.

DISCUSSION

Of the 5 ml (4.630 g) of cottonseed oil given to the rats, ca. 480 mg were recovered from the intestinal lavages and 905 mg from the feces of the adrenalectomized rats, whereas from the intact rats, 877 mg were recovered from the feces and 113 mg from the intestinal lavages. Thus the adrenalectomized rats absorbed ca. 3.245 g, whereas the intact rats absorbed ca. 3.640 g–a difference of ca. 400 mg.

This small difference in absorption is in agreement with results reported by Isselbacher (11). Furthermore Isselbacher noted that adrenalectomy significantly decreased the activity of the triglyceride synthetase system in the small intestinal mucosa. Thus it is possible that the rate of lipid transport across the small intestinal wall would be slower in the adrenalectomized rats. However the difference in triglyceride synthetase acitivity due to adrenalectomy does not affect significantly the total amount of triglyceride absorbed over a 24 hr period (Table II).

Isselbacher also noted that thiokinase activity in the microsomes of the small bowel mucosa of adrenalectomized rats was 50% lower than that found in intact rats. He also reported that decreases of a similar magnitude in the concentrations of monoglycerides and

diglycerides are found in the small bowel mucosa of adrenalectomized rats compared to intact rats. In the study reported here it was found (Table III) that the total glyceride concentration in the small intestinal wall was twice as high in the adrenalectomized rats as in the intact rats, $P < 0.05$. Since the total glycerides in this study were not subfractionated into mono-, di and triglycerides, and since Isselbacher did not report out total glycerides, it is not possible to compare the two sets of results in order to see whether the differences noted were due to the concentration of triglycerides.

It was further noted by Isselbacher, as well as by many other workers, that the resynthesis of triglyceride in the small intestinal mucosa involved activation of the fatty acid by thiokinase with the formation of a fatty acyl-CoA. The fatty acyl-CoA would then react in a transacylase-mediated reaction with α-glycerophosphate to form a lysophosphatidic acid and then a phosphatidic acid. Both lysophosphatidic acid and phosphatidic acid would assay as a phospholipid. From Table III it can be noted that the small intestinal mucosal phospholipid in the adrenalectomized rat is only 20% of that found in the intact rat, $P < 0.01$. This would be in agreement with the results reported by Isselbacher, where he shows a significantly lower phosphatidic acid pathway activity in the adrenalectomized rat compared with the intact rat.

Nevertheless the difference in total absorption between intact and adrenalectomized rats is only 400 mg out of a total of 4630 mg of triglyceride given to the animals. Thus it would appear that a triglyceride synthesis pathway other than the phosphatidic acid pathway was the major synthetic route in adrenalectomized rats. The monoglyceride pathway, in which no synthesis of phospholipids is required (12), could thus be the prime triglyceride synthesis pathway in adrenalectomized rats. This could account for the fact that adrenalectomized rats absorbed 90% of the ingested triglyceride absorbed by intact rats.

Various authors have shown that the circulating life of absorbed triglyceride is only a few minutes (13-17), and that between one third and half of the absorbed triglyceride is taken up quite rapidly by the liver (14, 18-20). If the liver took up the triglyceride and did not release it rapidly as pre-β-lipoproteins, then an accumulation of lipid in the liver must occur. Such situations have been reported by several investigators (21-25). From Figure 2, it can be seen that an accumulation of triglyceride occurred in the livers of the intact rats after 10 hr. This elevation occurred immediately following

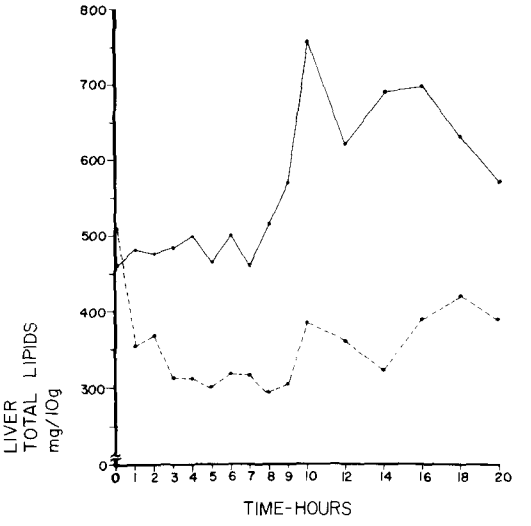

FIG. 3. Liver total lipid variation with time following a 5.0 ml cottonseed oil load in intact (——) and adrenalectomized (------) Long Evans rats.

the serum triglyceride elevation, indicative of clearance and temporary accumulation by the liver. The rise in liver triglycerides in the intact rats was absent in the adrenalectomized rats. Stern and Shapiro (26) reported that chylomicra fatty acids could be directly incorporated into adipose tissue without prior passage through the liver. This could be one possible explanation for the absence of a liver triglyceride rise in the adrenalectomized rats. In a previous report (27), it was noted that liver glycogen did not change in the fasted adrenalectomized rat in response to a triglyceride load. Thus lipids would have to be a major energy source. Their rapid oxidation could further account for the absence of a triglyceride rise in the livers of the adrenalectomized rats. The gradual decrease in liver free fatty acids in the adrenalectomized rats would tend to support this hypothesis.

The liver is a known source of phospholipid and bile acid synthesis. From Table I it can be seen that the fecal execretion of both phospholipids and bile acids are significantly lower in the adrenalectomized rats compared to the intact animals. It would appear that adrenalectomy significantly depressed liver phospholipid and bile acid synthesis. Liver phospholipids were significantly lower during the early hours in the adrenalectomized rats (Fig. 1) and did not significantly vary in response to a triglyceride load. This would support the concept of depressed liver phospholipid synthesis in the adrenalectomized rats.

Intestinal wall phospholipids in the adrenal-

ectomized rats were significantly lower than in the intact rats. This would support the concept of lowered triglyceride synthetase activity in adrenalectomized rats advanced by Isselbacher (11). It would appear that phospholipid synthesis in tissues is influenced by the adrenal gland.

It was noted (Table I) that there appeared to be a significant depression in intestinal triglyceride hydrolysis in adrenalectomized rats compared to intact controls. This would indicate decreased pancreatic lipase activity in the adrenalectomized rats. Fritz and Melius (28) reported that the lipolysis of triglycerides to diglycerides is enhanced by taurocholate while the lipolysis of diglyceride is depressed. They reported that this conjugated bile acid can split the lipase-diglyceride complex and simultaneously decrease the activation energy of the triglyceride lipolysis. The depressed bile acid production in the adrenalectomized rats (Table I) could explain the decreased triglyceride lipolysis noted in these rats. It might be expected that since taurocholate inhibits the lipolysis of diglyceride to monoglyceride, a larger relative percentage of monoglyceride to diglyceride should be seen in the adrenalectomized rats. This was not found. Fritz and Melius (28) did note that the effect of taurocholate was not linear with concentration. Thus the 50% decrease in fecal bile acids found in the adrenalectomized rats compared to intact controls need not be reflected in a 50% alteration of the monoglyceride to diglyceride ratio. Additionally, monoglyceride is quite readily absorbed and thus the ratio of monoglyceride to diglyceride found in the feces would not be expected to reflect the ratio of these glycerides in the small intestine during absorption.

Many questions remain to be answered. Among them are the identity of the adrenal hormone or hormones influencing bile acid production, the triglyceride synthetase system via the α-glycerophosphate route, and phospholipid metabolism and transport. In order to obtain answers to these questions, replacement therapy studies are required. Studies of this nature are being undertaken.

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REFERENCES

1. Shafrir, E., K.E. Sussman and D. Steinberg, J. Lipid Res. 1:459 (1960).
2. Salvador, R.A., K.I. Colville and J.J. Burns, Ann. N.Y. Acad. Sci. 131:113 (1965).
3. Barrett, A.M., J. Endocrinol. 36:301 (1966).
4. Lenz, P.H., A.I. Fleischman and T. Hayton, Lipids 4:115 (1969).
5. Lenz, P.H., G.H. Wien and A.I. Fleischman, Ibid. 5:524 (1970).
6. Ingle, D.J., and J.Q. Griffith, "The Rat in Laboratory Investigation," J.B. Lippincott Co., Philadelphia, Pa., 1942.
7. Fleischman, A.I., H. Yacowitz, T. Hayton and M.L. Bierenbaum, J. Nutr. 88:255 (1966).
8. Fleischman, A.I., T. Hayton, M.L. Bierenbaum and E. Wildrick, in "Automation in Analytical Chemistry," Proc. Technicon Symposium, New York, 1967, p. 21.
9. Schrade, W., E. Boehle, R. Biegler and E. Harmuth, Lancet i:285 (1963).
10. Dixon, W.J., and F.J. Massey, "Introduction to Statistical Analysis," New York, 1957, p. 287.
11. Isselbacher, K., in "Proc. 1967 Deuel Conference on Lipids," Edited by G. Cowgill and L.W. Kinsell, USDHEW, Washington, D.C., 1967, p.30.
12. Johnston, J., Ibid., p. 40.
13. Havel, R.J., and D.S. Fredrickson, J. Clin. Invest. 35:1025 (1956).
14. Olivecrona, T., J. Lipid Res. 3:439 (1962).
15. French, J.E., and B. Morris, J. Physiol. 138:326 (1957).
16. Nestel, P.J., M.A. Denborough and J. O'Dea, Circ. Res. 10:786 (1962).
17. Belfrage, P., B. Borgström and J. Olivecrona, Acta Physiol. Scand. 58:111 (1963).
18. Bragdon, J.H., and R.S. Gordon, Jr., J. Clin. Invest. 37:574 (1958).
19. French, J.E., and B. Morris, J. Physiol. 140:262 (1958).
20. Nestel, P.J., R.J. Havel and A. Berman, J. Clin. Invest. 41:1915 (1962).
21. Recknagel, R.O., and B. Lombardi, J. Biol. Chem. 236:564 (1961).
22. Lombardi, B., and R.O. Recknagel, Amer. J. Pathol. 40:571 (1962).
23. Maling, H.M., A. Frank and M.G. Horning, Biochim. Biophys. Acta 64:540 (1962).
24. Recknagel, R.O., B. Lombardi and M.C. Schote, Proc. Soc. Exp. Biol. Med. 104:608 (1960).
25. Heimberg, M., I. Weinstein, G. Dischmon and A. Dunkerley, J. Biol. Chem. 237:3623 (1962).
26. Stern, I., and B. Shapiro, Metabolism 3:539 (1954).
27. Lenz, P.H., and A.I. Fleischman, Fed. Proc. 28:515 (1969).
28. Fritz, P.J., and P. Melius, Can. J. Biochem. Physiol. 41:719 (1963).

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Damage to Microsomal Membrane by Lipid Peroxidation

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ABSTRACT

Alterations of microsomal membrane integrity were examined during lipid peroxidation. Loss of membrane-bound NADPH cytochrome *c* reductase activity and protein release into the aqueous phase were related to disruption of the lipophilic region of the membrane. Formation of fluorescent products in the lipid phase of the membrane occurred only in the presence of peroxidation products. Changes in the membrane lipid phase during peroxidation included a decrease in the reactive amino groups of the phospholipids and a decrease in detectable phosphatidyl ethanolamine. In an ultrafiltration continuous flow chamber, peroxidized microsomal membranes were hydrolyzed to a lesser degree by solubilized lysosomal cathepsins and to a greater degree by lysosomal nucleases than were nonperoxidized membranes.

INTRODUCTION

Biological membranes, especially those of subcellular organelles, are labile to lipid peroxidation because of their high content of polyunsaturated lipids (1). Among the products of oxidative deterioration of these unsaturated lipids are free radical intermediates, semistable peroxides and reactive carbonyls (2). The mechanisms by which cellular systems are damaged include free radical polymerization (3,4), aldehyde crosslinking polymerization (5,6) and disruption of membrane lipid-protein integrity (7). Alterations in phospholipid structure also occur during peroxidation of purified phospholipids (8,9) and of complex phospholipids of membranes (10,11).

Several models for membrane structure, including the interaction between lipid-protein complexes, have been discussed by Branton and Deamer (12). The importance of examining changes in structural integrity caused by lipid peroxidation is apparent. An understanding of these changes would enable a better understanding of the pathological conditions induced by lipid peroxidation in vivo. Several pathological disorders may involve lipid peroxidation damage, including some phases of atherosclerosis (13,14), aging (15,16), neuronal ceroid-lipofuscinosis or Batten's syndrome (17) and liver injury caused by ethanol (18) or chlorinated hydrocarbons (19). The microsomal membrane was chosen as a model system to evaluate alterations in membrane integrity induced by lipid peroxidation.

MATERIALS AND METHODS

Source of Materials

The materials obtained from Sigma Chemical Co., St. Louis, Mo., included 2-thiobarbituric acid (TBA), D glucose-6-phosphate, cytochrome *c* (horse heart), NADPH and dithioerythritol. 1,1,3,3-Tetraethoxypropane bis (diethyl acetal) was purchased from J.T. Baker Chemical Co., Phillipsburg, N.J. Butylated hydroxytoluene (BHT) was obtained from Eastman Chemical Co., Kingsport, Tenn. Precoated Silica Gel G thin layer chromatography (TLC) plates were purchased from Quantum Industries, Fairfield, N.J. The phospholipid standards, phosphatidyl ethanolamine and phosphatidyl choline, were purchased from Supelco, Inc., Bellefonte, Pa.; and phosphatidyl serine, phosphatidyl inositol, lysolecithin and sphingomyelin from Schwarz/Mann, Orangeburg, N.Y. The Diaflo ultrafiltration apparatus and ultrafilters (UM-2) were obtained from Amicon Corporation, Lexington, Mass.

Preparation of Subcellular Organelles

Rat liver was fractionated into nuclear, mitochondrial, light mitochondrial-lysosomal and microsomal fractions by the method of de Duve et al. (20) after homogenization in 250 mM sucrose and 1 mM EDTA.

Microsomal Peroxidation System

Aliquots of 25 mg microsomal membrane protein were suspended in 10 ml 10 mM Tris-HCl buffer, pH 7.4, in 50 ml flasks. Peroxidation was initiated by addition of 0.5 ml each of 10 mM ascorbate and 10 mM $FeCl_3$. The sealed flasks were shaken at 225 rpm in oxygen at 37 C. Flasks were removed for analyses of contents at 0, 2, 4, 6, 8, 10, 12, 18 and 24 hr. Controls contained in addition 1% BHT.

Analytical Methods

Lipid peroxidation was followed by the measurement of TBA reactive substances (TBRS). Aliquots of 0.5 ml of the microsomal suspension, 0.5 ml distilled water and 0.5 ml

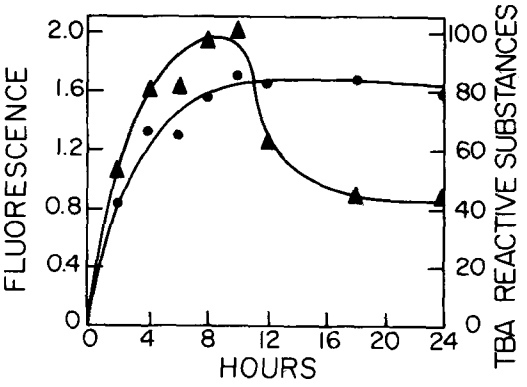

FIG. 1. Formation of fluorescent pigments (•) and production of TBA reactive substances (▲) during peroxidation of microsomal membranes. TBA reactive substances are expressed as nmoles of MA per milligram membrane protein. Fluorescence is expressed as the fluorescence times meter multiplier setting per milligram membrane protein.

30% trichloroacetic acid were mixed and centrifuged at ca. 3000 rpm for 3 min. The supernatant was added to an equal volume of aqueous 0.67% TBA, heated in a boiling water bath for 15 min, cooled to room temperature, and the absorbance measured at 532 nm was expressed as nanomoles of malonaldehyde (MA). The MA standard was prepared from 1,1,3,3-tetraethoxypropane bis (diethyl acetal) (21).

Extraction for measurement of fluorescence was done as follows. Aliquots of 2.5 ml microsomal suspensions were homogenized with 5.0 ml chloroform-methanol 2:1 for 1 min at room temperature. After addition of 2.5 ml distilled water the slurry was mixed for 15 sec and briefly centrifuged. Fluorescence spectra of the chloroform layers were recorded with an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Inc., Silver Spring, Md.). The slit arrangement was 3, 1 and 3 mm for slits 3, 4 and 6, respectively; the sensitivity was set at 50 and the meter multiplier was set in accordance with the fluorescence intensity of each sample. The quinine sulfate standard (1 μg/ml 0.1 N H_2SO_4) had a fluorescence intensity of 70 at a meter multiplier setting of 0.3.

Phospholipids were quantitated (22) and expressed as micromoles of inorganic phosphate (Pi). A 1.0 ml aliquot of the chloroform extract was concentrated and applied to a Silica Gel G plate. The phospholipids were separated by chloroform-methanol-water 80:25:3 and detected by iodine vapor. The fractions were removed from the TLC plates, hydrolyzed (22) and Pi determined.

Free amino groups of the phospholipids were determined by ninhydrin analysis on 0.5 ml aliquots of chloroform extracts after drying under nitrogen. The absorbance was measured at 570 nm and expressed as nanomoles of ethanolamine.

The protein released from the membrane during peroxidation was determined by the method of Miller (23) after centrifugation of 2.5 ml of the microsomal suspension at 100,000 x g for 1 hr.

NADPH cytochrome *c* reductase was assayed by addition of a 0.1 ml aliquot of the microsomal suspension to a spectrophotometer cuvette that contained 0.2 ml NADPH (1 mg/ml), 0.1 ml oxidized cytochrome *c* (10 mg/ml) and 1.6 ml 10 mM Tris-HCl buffer, pH 7.4. Absorbance was recorded at 550 nm for 3 min against a cytochrome *c* blank (24). Glucose-6-phosphatase was assayed by the method of Dallner (25), and the released Pi was measured by the method of Fiske and Subba-Row (26).

Preparation of Soluble Lysosomal Enzyme Fraction

The light mitochondrial-lysosomal fraction, resuspended in a small volume of 10 mM sodium acetate buffer, pH 5.4, was frozen and thawed 15 times to release the lysosomal enzymes. The membrane was removed by centrifugation at 100,000 x g for 60 min. The supernatant, containing the solubilized lysosomal enzymes, was dialyzed against 10 mM acetate buffer for 20 hr at 2 C with three changes of buffer. The transpeptidase activity of cathepsin C (27) in this preparation, which was used in the hydrolysis experiments, was 1525 nmoles of tyrosyl hydroxamate per minute per milligram of protein.

Hydrolysis of Peroxidized Microsomal Membrane

A 2.0 ml aliquot of the microsomal suspension (5.0 mg protein) and 1.0 ml of the lysosomal enzyme preparation (2.95 mg protein) were added to 7.0 ml 0.1 M sodium acetate buffer, pH 5.4, that contained 10 mM dithioerythritol. The mixture was placed in a 10 ml Diaflo-ultrafiltration apparatus with a UM-2 ultrafilter, which allowed molecules of less than 1000 mol wt to pass through. The reaction system was placed under nitrogen pressure to ensure a constant flow of buffer into and of hydrolysate out of the reaction chamber. A reaction temperature of 37 C and a flow rate of 2.0 ml/hr were maintained. The hydrolysate (1.65 ml fractions) was examined for products released by the cathepsin system (28) and acid nucleases (29). The products of proteolysis were determined with ninhydrin and the acid nuclease products were measured with orcinol (30).

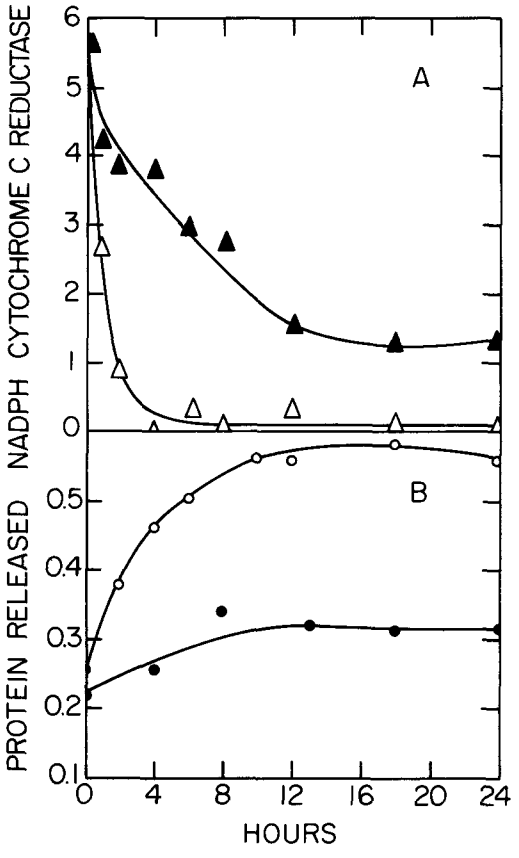

FIG. 2. (A) Loss of NADPH cytochrome *c* reductase activity in the absence (Δ) and presence (▲) of antioxidant during peroxidation of microsomal membrane. Activity is expressed as nmoles of cytochrome *c* reduced per minute per milligram protein. (B) Release of protein from microsomal membrane in the absence (○) and presence (●) of antioxidant during lipid peroxidation. Protein released is expressed as milligram solubilized protein per milligram remaining membrane protein.

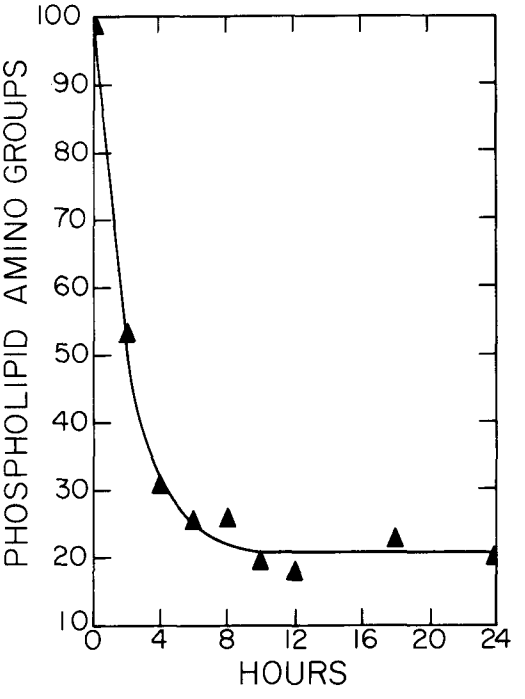

FIG. 3. Decrease in free amino groups of phospholipids during microsomal membrane peroxidation. The free phospholipid amino groups are expressed as percentage of the initial value in nmoles ethanolamine per μmole P_i.

Membrane remaining in the reaction chamber after hydrolysis by lysosomal enzymes was recovered by centrifugation at 100,000 x g for 60 min. Protein was determined on an aliquot; the remainder was extracted with chloroform-methanol and examined for fluorescence.

RESULTS

Microsomal membrane was chosen as a model to evaluate alterations in membrane integrity resulting from lipid peroxidation. The microsomes contain a complete complement of phospholipids and enzyme activities which can be examined. Addition of antioxidant, 1% BHT, to the microsomal suspension protected the membrane from peroxidation. These samples served as controls and allowed differentiation of changes in membrane integrity due to temperature and those changes caused by lipid peroxidation.

Figure 1 shows amounts of TBRS and fluorescent products as a function of time. TBRS increased for 8-10 hr and then decreased. Fluorescent products developed similarly until TBRS decreased, whereupon fluorescence development plateaued. All fluorescent products had maximum emission at 430 nm when excited at its maximum of 360 nm. In the presence of antioxidant the microsomes showed neither the formation of TBRS nor the development of significant fluorescence.

To evaluate the effect of lipid peroxidation on the activity of membrane-bound enzymes, NADPH cytochrome *c* reductase and glucose-6-phosphatase were measured. Peroxidizing membrane lost 50% of its initial NADPH cytochrome *c* reductase activity within the 1st hr (Fig. 2A). The antioxidant protected system lost activity also, but at a much slower rate. However loss of glucose-6-phosphatase activity was much more rapid and was not protected by antioxidant. Within 1 hr each sample contained less than 10% of its initial activity. The increased protein content of the microsomal supernatant (Fig. 2B) indicates that disruption

TABLE I

Phospholipid Composition of Peroxidized Microsomal Membrane

Peroxidation time, hr	Percentage of total phospholipid		
	Phosphatidyl ethanolamine	Phosphatidyl choline	Other[a]
0	22.0	66.6	11.4
2	12.6	60.6	26.8
4	8.2	60.2	31.7
6	12.4	60.6	27.8
8	11.8	57.7	30.8
10	6.5	60.8	36.0
12	6.8	57.5	35.8
18	3.1	59.8	38.7
24	2.6	60.6	37.0
24 (Control)	16.7	67.3	17.0
Literature[b]	24.0	64.0	12.0

[a]Phospholipids with an R_f less than 0.20, including phosphatidyl serine, phosphatidyl inositol, lysolecithin and sphingomyelin.

[b]See Reference 11.

of the membrane occurred. Added antioxidant partially stabilized the membrane and decreased the release of protein from the membrane. The results of these studies on peroxidizing membrane indicated that the lipophilic portion of the membrane was disrupted, which decreased the interaction between the enzyme and the membrane lipids.

Alterations in the lipid phase were examined by analysis of the phospholipid extract. During peroxidation the amount of extractable phospholipid decreased, with a larger decrease in amino phospholipids (Fig. 3). In the presence of antioxidant these alterations did not occur.

The percentage of each phospholipid fraction is shown in Table I as a function of peroxidation time. The lower R_f phospholipids were combined, since they were not as clearly separated from each other as were phosphatidyl ethanolamine and phosphatidyl choline. The trend observed was that the phosphatidyl ethanolamine fraction decreased with increased peroxidation, while the percentage of the lower R_f phospholipids increased.

Using the Diaflo ultrafiltration apparatus as a reaction chamber, microsomal membrane damaged by peroxidation for various intervals of time was hydrolyzed by lysosomal enzymes. Rates of hydrolysis appeared to be first order. The results of these experiments are best shown by the total products of hydrolysis given in Table II. Two indicators of lysosomal hydrolysis were measured: catheptic activity, which releases amino acids and peptides, and acid nuclease activity, which releases nucleotide fragments from the membranes. Results are expressed in terms of standards as micromoles of leucine and micromoles of adenosine. A decrease in hydrolyzability of the protein part of the peroxidized membrane is indicated by the accumulation of products released by cathepsin hydrolysis, while the products released by acid nuclease hydrolysis suggest increased hydrolyzability of the nucleic acids. Although both of these trends seem apparent, they are not statistically significant. Examination of hydrolysate fractions for aqueous fluorescent damage products, which could have been released during hydrolysis of the peroxidized membrane, was negative. Only the natural fluorescence of the aromatic amino acids was observed.

The chloroform-methanol extract of the residual membrane after 12.8 hr of hydrolysis was examined for accumulated fluorescent products. The fluorescence spectra were similar to those of the fluorescent products measured as a function of peroxidation time (Fig. 1) and to that of lipofuscin pigments (2). Maximum fluorescence was at 430 nm when excited at 360 nm.

The concentration of fluorescent products in the microsomal membrane was examined before and after lysosomal hydrolysis. The nonperoxidized membrane (0 hr) had an increase in fluorescence from less than 0.1 to 1.3 fluorescence units per milligram membrane protein during hydrolysis. It apparently underwent peroxidation during the hydrolysis reaction. The peroxidized membrane samples of 4-24 hr had higher initial fluorescence but increased from 0.6 to 0.9-1.1 fluorescence units per milligram membrane protein. In both cases the fluorescent pigment concentration was greater after hydrolysis.

DISCUSSION

The microsomal membrane has been used in

TABLE II

Products of Peroxidized Microsomal Membranes Hydrolyzed by Lysosomal Enzymes

Time of peroxidation, hr	Amino acids, mmol[a]	Nucleotides, μmol[a]
0	0.30	9
4	0.23	13
8	0.21	12
12	0.16	12
18	0.15	15
24	0.21	12

[a]Total moles of product released from 5 mg microsomal membrane during 12.8 hr hydrolysis with 2.95 mg lysosomal enzymes in 10 ml 100 mM sodium acetate buffer, pH 5.4.

many laboratories as a model in studies of lipid peroxidation (7,10,11,31-33), and antioxidants have been used to protect membrane systems from peroxidative damage (34-37). The lack of formation of TBRS in the presence of added BHT indicates inhibition of lipid peroxidation. In microsomal membranes the formation of fluorescent products with a fluorescence maximum of 430 nm and an excitation maximum at 360 nm occurred only when the membranes underwent lipid peroxidation. This was noted earlier for peroxidation of amino phospholipids (34) and for the organelle membranes examined for dietary antioxidant protection (33).

The accumulation of TBRS in the peroxidizing membrane results from the formation of aldehydes and ketones. The eventual decline of TBRS is due to a decrease in the available unsaturated lipid content; to the reaction of TBRS, especially MA, with amino groups of amino acids, proteins or amino phospholipids; to the further oxidative decomposition of TBRS; and to condensation reactions in which TBRS form unreactable polymers.

The loss of membrane-associated enzyme activity is most likely the result of a combination of damaging processes, including free radical polymerization (3), aldehyde polymerization (6) and disruption of lipid-protein interaction (7). At the membrane surface, disruption of lipophilic sites by peroxidation simulates a detergent effect in the inactivation of enzyme systems (7). The observation of increased protein release into the microsomal supernatant during peroxidation strongly supports this theory. The fact that glucose-6-phosphatase activity was lost much more rapidly than the NADPH cytochrome *c* reductase activity suggests that the latter enzyme system is more tightly bound to the membrane. These experiments show that the integrity of the membrane is dependent upon the retention of lipid-protein interaction.

A decrease in the phosphatidyl ethanolamine content and the loss of reactive amino groups during peroxidation indicates the formation of aldehyde-amine reaction products, some of which are fluorescent. During peroxidation of phosphatidyl ethanolamine, a decrease in free amino groups was shown (8) and Schiff base products were formed between aliphatic aldehydes and the amino group of the phospholipid (9).

Decrease of phosphatidyl ethanolamine, with an equal and concomitant increase in the lower R_f phospholipid fractions, is in accord with preliminary studies with a dipalmityl phosphatidyl ethanolamine-malonaldehyde model system wherein the reaction products migrated at a lower R_f than the initial phosphatidyl ethanolamine (34). Although this evidence does not prove the formation of any particular chromophoric group, it does suggest the formation of modified phosphatidyl ethanolamine with a reduced R_f.

How do alterations in membrane structure like these affect their degradation and turnover by the lysosomal enzyme system? The theory for the formation of lipofuscin pigments involves ingestion of membrane components which cannot be completely hydrolyzed by the lysosome. The lysosome does not release these products and they accumulate as lipofuscin pigment (38). The fluorescence of lipofuscin pigments has been tentatively identified as characteristic of a conjugated Schiff base formed by the reaction of malonaldehyde with two amino compounds and having a 1-amino-3-iminopropene structure (39). A model system was used to mimic the lysosome and to evaluate the effect of peroxidative damage on the hydrolysis of microsomal membrane. Since the protein released into the soluble phase increased with peroxidation damage, a greater rate of hydrolysis by lysosomal enzymes might be expected. A possible explanation for the apparent decrease in hydrolysis is that the

released protein formed aggregates, by interaction of their lipophilic regions, which could not be hydrolyzed easily by the lysosomal enzymes. Aggregation of microsomal suspensions during peroxidation has been described (31). Increased release of nucleotides probably resulted from hydrolysis of released ribosomal subunits and *m*-RNA. Arstila et al. (40) showed by electron microscopy that increased peroxidation caused detachment of ribosomes from membrane followed by dissociation of the ribosomal particles. The structure of the microsome then changed from round to less regular vesicles and finally to a dense, amorphous precipitate that contained membrane debris.

After hydrolysis of the peroxidized microsomes, the apparent increase in fluorescent pigment concentration was partially caused by a decrease in the protein concentration during hydrolysis by lysosomal enzymes and ultrafiltration. The nonperoxidized membrane (0 hr) had a greater increase in fluorescence than the peroxidized membranes due to the continued peroxidation of the microsomal membrane during the lysosomal hydrolysis. In vivo, the same result might be expected. Once initiated, membrane peroxidation could continue even during the degradation process within the lysosome.

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REFERENCES

1. Fleischer, S., and G. Rouser, JAOCS 42:588 (1956).
2. Tappel, A.L., in "Pathological Aspects of Cell Membranes," Vol. 1, Edited by B.F. Trump and A. Arstila, Academic Press, New York, In press.
3. Roubal, W.T., and A.L. Tappel, Arch. Biochem. Biophys. 113:150 (1966).
4. Roubal, W.T., Lipids 6:62 (1971).
5. Menzel, D.B., Ibid. 2:83 (1967).
6. Chio, K.S., and A.L. Tappel, Biochemistry 8:2827 (1969).
7. Wills, E.D., Biochem. J. 123:983 (1971).
8. Corliss, G.G., and L.R. Dugan, Jr., Lipids 5:846 (1970).
9. Rao, G.V., and L.R. Dugan, Jr., JAOCS 47:289 (1970).
10. May, H.E., and P.B. McCay, J. Biol. Chem. 243:2288 (1968).
11. Victoria, E.J., and A.A. Barber, Lipids 4:582 (1969).
12. Branton, D., and D.W. Deamer, Protoplasmatologia II/E/1: 1 (1972).
13. Fujii, T., and T. Sato, Chem. Pharm. Bull. (Tokyo) 19:385 (1971).
14. Perkins, E.G., T.H. Joh and F.A. Kummerow, in "Metabolism of Lipids as Related to Atherosclerosis," Edited by F.A. Kummerow, C. Thomas, Springfield, 1965, p. 48.
15. Harman, D., J. Amer. Geriat. Soc. 17:721 (1969).
16. Tappel, A.L., Geriatrics 23:97 (1968).
17. Zeman, W., Advan. Gerontol. Res. 3:147 (1971).
18. DiLuzio, N.R., and A.D. Hartman, Exp. Mol. Pathol. 11:38 (1969).
19. Recknagel, R.O., Pharm. Rev. 19:145 (1967).
20. de Duve, C., B.C. Pressman, R. Gianetto, R. Wattiaux and F. Appelmans, Biochem. J. 60:604 (1955).
21. Kwon, T.W., and B.M. Watts, J. Food Sci. 28:627 (1963).
22. Rouser, G., S. Fleischer and A. Yamamoto, Lipids 5:494 (1970).
23. Miller, G.L., Anal. Chem. 31:964 (1958).
24. Horecker, B.L., in "Methods in Enzymology," Vol. II, Edited by S.P. Colowick and N.O. Kaplan, Academic Press, New York, 1955, p. 704.
25. Dallner, G., Acta Pathol. Microbiol. Scand. Suppl. 166:1 (1963).
26. Fiske, C.H., and Y. SubbaRow, in "Methods in Enzymology," Vol. III, Edited by S.P. Colowick and N.O. Kaplan, Academic Press, New York, 1957, p. 843.
27. Metrione, R.M., A.G. Neves and J.S. Fruton, Biochemistry. 5:1597 (1966).
28. Ansorge, S., P. Bohley, H. Kirschke, J. Langner, and H. Hanson, Acta Biol. Med. Ger. 26:953 (1971).
29. Futai, M., S. Mirjata, and D. Mizuno, J. Biol. Chem. 244:4951 (1969).
30. Litwack, G., in "Experimental Biochemistry," John Wiley and Sons, 1966, p. 21.
31. Hochstein, P., and L. Ernster, in "CIBA Symposium on Cellular Injury," Edited by A.V.S. de Reuck and J. Knight, Little and Brown, Boston, 1964, p. 123.
32. Robinson, J.D., Arch. Biochem. Biophys. 112:170 (1965).
33. Dillard, C.J., and A.L. Tappel, Lipids 6:715 (1971).
34. Bidlack, W.R., Ph.D. thesis, University of California, Davis, 1972.
35. Tappel, A.L., Ann. N.Y. Acad. Sci. 203:12 (1972).
36. Gruger, E.H., and A.L. Tappel, Lipids 6:147 (1971).
37. Tinberg, H.M., and A.A. Barber, J. Nutr. 100:413 (1970).
38. Porta, E.A., and W.S. Hartroft, in "Pigments in Pathology," Edited by M. Wolman, Academic Press, New York, 1969, p. 191.
39. Chio, K.S., and A.L. Tappel, Biochemistry 8:2821 (1969).
40. Arstila, A.U., M.A. Smith, and B.F. Trump, Science 175:530 (1972).

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Fluorescent Products from Reaction of Peroxidizing Polyunsaturated Fatty Acids with Phosphatidyl Ethanolamine and Phenylalanine

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ABSTRACT

Fluorescent chromophores produced by reaction of peroxidizing arachidonic acid or methyl docosahexaenoate with synthetic dipalmityl phosphatidyl ethanolamine were lipid soluble, and those from reaction with phenylalanine were water soluble. In all reaction systems that contained polyunsaturated fatty acid and only one amine compound, the development of fluorescence was linearly related to oxygen absorption for 12-24 hr ($p<0.001$) and to the amount of thiobarbituric acid reacting materials until the rate of oxygen absorption decreased. The fluorochromes typically had maximum excitation at 360 nm and maximum emission at 430-440 nm, indicating that they were conjugated Schiff bases with the general structure R-N=C-C=C-N-R, where R represents the amino acid phenylalanine or the phospholipid phosphatidyl ethanolamine. The fluorochromes were similar to those extracted from isolated age pigments and tissues of animals that are aged, vitamin E-deficient, or stressed with highly unsaturated lipid diets.

INTRODUCTION

The production of water soluble fluorescent compounds in subcellular organelles in vitro is a function of time of peroxidation (1). Absorption of oxygen and production of thiobarbituric acid (TBA) reactants and lipid soluble fluorescent products in peroxidizing rat liver mitochondria and microsomes also occur as a function of time and are inversely related to dietary α-tocopherol levels (2). Water soluble fluorescent products derived from reaction of malonaldehyde with amino acids have been synthesized and characterized, and the chromophoric system R-N=C-C=C-N-R is responsible for the fluorescence (3). The lipid soluble fluorescent chromophores from animal tissues have been described as probably being derived from the reaction of malonaldehyde with the amine groups of phospholipids (4,5). Fluorescent compounds extracted from isolated age pigments (4) have spectral characteristics similar to those of chromophores extracted from peroxidizing subcellular organelles (2) and of chromophores formed in this study from the reaction between phosphatidyl ethanolamine and peroxidizing polyunsaturated fatty acids.

This study was done to relate lipid soluble fluorescent chromophores derived from phosphatidyl ethanolamine to those found in other reactions in vitro (1,2) and to those associated in vivo with aging (6) and with vitamin E deficiency (5); to compare the rates of development of aqueous and lipid soluble fluorescent products in reaction systems that contained peroxidizing polyunsaturated fatty acid and amines; and to show the correlations of fluorescence development with oxygen absorption and with formation of TBA reactants.

EXPERIMENTAL PROCEDURES

Materials

Arachidonic acid was obtained from Hoff-

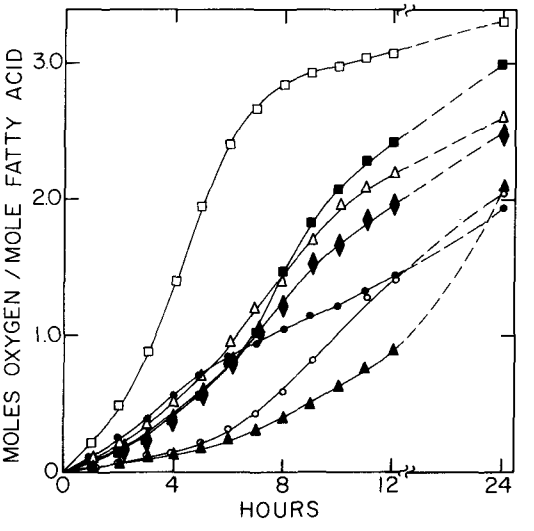


FIG. 1. Absorption of oxygen by (▲) ethyl arachidonate; (●) arachidonic acid and phenylalanine; (△) ethyl arachidonate and dipalmityl phosphatidyl ethanolamine; (□) methyl docosahexaenoate and dipalmityl phosphatidyl ethanolamine; (■) methyl docosahexaenoate, (○) methyl docosahexaenoate and phenylalanine; (◆) ethyl arachidonate, dipalmityl phosphatidyl ethanolamine and phenylalanine.

TABLE I

Effect of Phosphatidyl Ethanolamine or Phenylalanine on TBA-Reacting Material[a] during Peroxidation of Unsaturated Fatty Acids

Time, hr	20:4[b]	20:4 + PE	20:4 + Phe	20:4 + PE + Phe	22:6	22:6 + PE	22:6 + Phe
0	2.5	5.4	1.0	11.4	10.0	5.1	1.2
2	3.5	19.1	5.8	17.4	25.2	40.1	2.1
4	3.9	44.1	12.4	27.6	79.4	125.0	5.1
6	6.1	85.1	16.6	46.7	133.0	183.4	11.4
8	25.2	122.4	14.4	77.4	130.4	118.5	18.9
10	41.6	121.0	17.1	95.8	52.5	90.1	28.9
12	25.5	---	15.0	99.9	33.9	63.3	27.6
24	29.2	43.5	14.3	96.5	20.0	28.8	27.2

[a]Millimoles TBA reactants per mole unsaturated fatty acid; average of duplicates.

[b]20:4, Ethyl arachidonate; 22:6, methyl docosahexaenoate; PE, synthetic dipalmityl phosphatidylethanolamine; Phe, phenylalanine.

man-LaRoche, Nutley, N.J., and its esters as well as methyl docosahexaenoate were purchased from the Hormel Institute, Austin, Minn. Synthetic dipalmityl phosphatidyl ethanolamine was purchased from Schwarz/Mann, Orangeburg, N.J.; phenylalanine from Nutritional Biochemicals Corp., Cleveland, Ohio; and 2-thiobarbituric acid from Sigma Chemical Co., St. Louis, Mo. Spectral quality methanol and chloroform were obtained from Mallinckrodt, St. Louis, Mo. All other chemicals and biochemicals were reagent or research grade and were obtained from a variety of commercial sources.

Analytical Methods

Peroxidation of arachidonate and docosahexaenoate was followed by measurements of oxygen absorption, TBA reactants and fluorescence. Oxygen absorption was measured by the Warburg manometric method. Separate reaction flasks were used for each time period at which the TBA reaction and fluorescence analyses were done. Oxygen absorption at 37 C was recorded every hour for 12 or 14 hr and again at 24 hr. The 2.5 ml reaction mixtures consisted of 0.1 M potassium phosphate buffer, pH 7.0, 10^{-5} M ferric chloride, 10^{-5} M ascorbic acid, 40 mM fatty acid, and in the appropriate flasks 40 mM phenylalanine and/or 40 mM synthetic dipalmityl phosphatidyl ethanolamine. Emulsions of fatty acid with phosphatidyl ethanolamine or phenylalanine were made by homogenizing the reactants with a motor-driven glass and Teflon homogenizer; some emulsions were aided by sonication with a sonic dismembrator (Quigley-Rochester Inc., Rochester, N.Y.) for 10-30 min. After homogenization the emulsions were adjusted to pH 7 with 1 N KOH. The reaction was initiated by addition of ascorbic acid and ferric chloride from side arms of the Warburg vessels.

The TBA test was done essentially according to the method of Wills (7). Aliquots of the oxidized fatty acid reaction mixtures were diluted with water to 1 ml; 1 ml of 20% w/v trichloroacetic acid and 2 ml of 0.67% w/v 2-thiobarbituric acid were added and the tubes placed in a boiling water bath for 15 min. The absorbance of the cooled and centrifuged samples was read at 532 nm. An extinction coefficient of 1.56×10^5 was used for calculations.

For fluorescence measurements aliquots of the oxidizing reaction mixtures were diluted to 1 ml with water and then extracted at room temperature with 2 ml of chloroform-methanol 2:1 v/v by mixing for 2 min on a vortex mixer at high speed. After centrifugation for 2-3 min, the water-methanol and chloroform layers were separated. When necessary, further dilution of these extracts was made. Complete fluorescence spectra were determined with an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Inc., Silver Spring, Md.). The slit arrangement for recording fluorescence spectra was slits 3, 4 and 6, set at 3, 1 and 3 mm, respectively. The sensitivity setting was 50. Spectra were recorded on an X-Y recorder (Houston Instrument, Bellaire, Tex.). With the above instrument settings, 1 μg standard quinine sulfate per milliliter 0.1 N H_2SO_4 had a fluorescence intensity of 60 at a 0.3 meter multiplier setting; corrections were made for variations in quinine sulfate fluorescence due to Xenon lamp fluctuations.

RESULTS

Absorption of Oxygen

Figure 1 presents the curves for absorption

TABLE II

Linear Regression Correlation Coefficients and Statistical Probabilities for Data Relating Absorption of Oxygen, TBA Reactants and Fluorescence

Reaction system	Variables	Time period, hr	r	p
20:4 + Phe	Aqueous fluorescence Oxygen absorbed	0-24	0.99	<0.001
	Aqueous fluorescence TBA reactants	0-6	0.97	<0.05
	TBA reactants Oxygen absorbed	0-10	0.95	<0.01
20:4 + PE	Lipid soluble fluorescence Oxygen absorbed	0-24	0.99	<0.001
	Lipid soluble fluorescence TBA reactants	0-8	1.0	<0.001
	TBA reactants Oxygen absorbed	0-8	1.0	<0.001
20:4 + PE + Phe	Aqueous fluorescence Oxygen absorbed	0-12	0.97	<0.001
	Lipid soluble fluorescence Oxygen absorbed	0-24	0.93	<0.001
	Aqueous fluorescence TBA reactants	0-12	0.95	<0.01
	Lipid soluble fluorescence TBA reactants	0-12	0.94	<0.01
	TBA reactants Oxygen absorbed	0-12	0.99	<0.001
22:6 + PE	Lipid soluble fluorescence Oxygen absorbed	0-24	0.98	<0.001
	Lipid soluble fluorescence TBA reactants	0-6	0.99	<0.01
	TBA reactants Oxygen absorbed	0-6	0.99	<0.01
22:6 + Phe	Aqueous fluorescence Oxygen absorbed	0-24	1.00	<0.001
	Aqueous fluorescence TBA reactants	0-10	0.98	0.001
	TBA reactants Oxygen absorbed	0-12	0.96	<0.001

of oxygen as a function of time up to 24 hr. Ethyl arachidonate had the longest induction period, which was overcome to different degrees in the presence of phosphatidyl ethanolamine or phenylalanine, or both. By 24 hr ca. 2 mol oxygen were absorbed per mole of fatty acid with and without phenylalanine, and ca. 2.4 mol in the presence of phosphatidyl ethanolamine. The dipalmityl phosphatidyl ethanolamine control did not absorb oxygen by 24 hr. A similar effect on the induction period occurred during oxidation of methyl docosahexaenoate in the presence of phosphatidyl ethanolamine. By 24 hr each mole of this fatty acid absorbed ca. 3 mol oxygen, and in the presence of phosphatidyl ethanolamine ca. 3.3. mol. The induction period for peroxidizing methyl docosahexaenoate in the presence of phenylalanine was greater than that for the fatty acid alone, and the amount of oxygen absorbed by 24 hr was only 2 mol per mole of fatty acid.

TBA-Reacting Materials

Table I gives the amounts of TBA-reacting materials in the various peroxidizing systems measured over a 24 hr time course. The presence of phosphatidyl ethanolamine increased the production of TBA-reactants from oxidizing arachidonate; phenylalanine caused a considerably smaller increase. The increase in TBA-reactants from oxidizing docosahexaenoate was less in the presence of phosphatidyl ethanolamine than for arachidonate in a similar reaction. Phenylalanine considerably decreased the production of TBA reactants from methyl docosahexaenoate. Although the level of TBA reactants in the different reaction systems varied, Table II shows that TBA reactants were significantly and highly correlated with absorption of oxygen for 6-12 hr. The time period over which there was correlation was dependent approximately upon when the rate of oxygen absorption decreased, as indicated in the curves for oxygen absorption (Fig. 1).

Production of Fluorescent Chromophores

Figure 2 shows fluorescence spectra of chromophores produced as a function of three

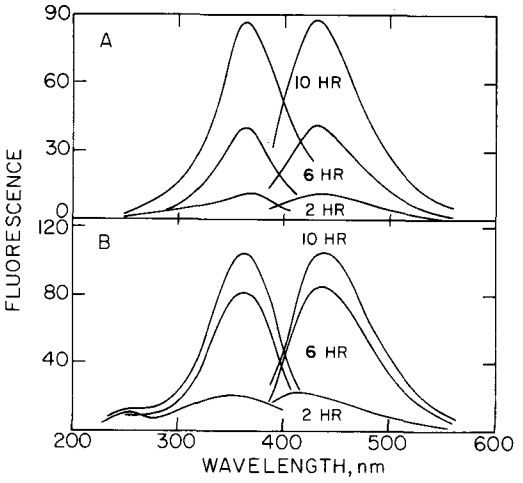


FIG. 2. Fluorescence spectra of (A) chloroform-methanol extracts from ethyl arachidonate-phosphatidyl ethanolamine reaction mixtures and of (B) aqueous extracts from arachidonic acid-phenylalanine reaction mixtures at 2, 6 and 10 hr. The meter multiplier setting was 0.3. Fluorescence represents that produced per micromole of peroxidizing fatty acid. Extraction procedures and other instrument parameters are described in the text.

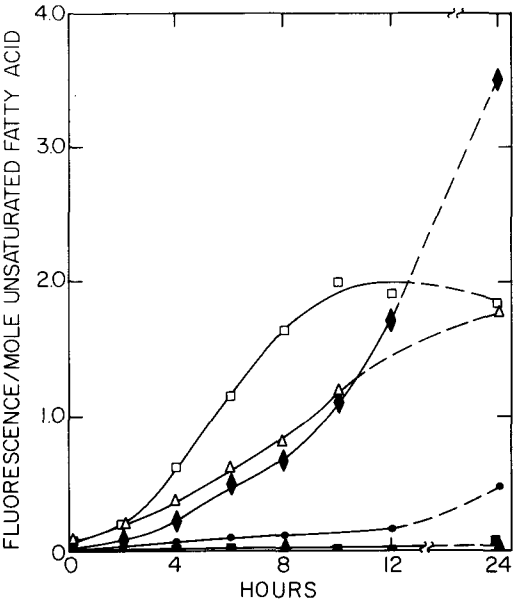


FIG. 3. Formation of lipid soluble fluorescent products as a function of time. Extraction procedures are described in the text. Fluorescence is expressed as mmoles equivalent glycine-1-amino-3-iminopropene standard fluorescence. Excitation and emission maxima for 10 hr samples are given. (▲) Ethyl arachidonate, 360 nm, 425 nm; (△) ethyl arachidonate and phosphatidyl ethanolamine, 360 nm, 430 nm; (◆) ethyl arachidonate, phosphatidyl ethanolamine and phenylalanine, 365 nm, 430 nm; (●) arachidonic acid and phenylalanine, 390 nm, 470 nm; (■) methyl docosahexaenoate, 370 nm, 450 nm; (□) methyl docosahexaenoate and phosphatidyl ethanolamine, 360 nm, 430 nm. Phosphatidyl ethanolamine was not fluorescent.

reaction times during the oxidation of ethyl arachidonate in the presence of phosphatidyl ethanolamine and phenylalanine. The excitation maxima at 360 nm and emission maxima at 430-440 nm are typical of the fluorescent products studied. The fluorescence maxima varied slightly with solvent, concentration, reactants and oxidation time. Complete fluorescence spectra were taken of samples from all reaction systems at 2 hr intervals. Figure 3 shows the formation of lipid soluble fluorescent products in the various peroxidizing systems as a function of time up to 24 hr. Figure 4 shows the formation of aqueous fluorescent products, primarily in those systems that contained phenylalanine. Peroxidizing arachidonic acid reacted with phenylalanine produced primarily aqueous fluorescent products; the lipid soluble fluorescent products at 12 and 24 hr were 13% and 24%, respectively, of the aqueous fluorescent products. Methyl docosahexaenoate reacted with phosphatidyl ethanolamine produced mainly lipid soluble fluorescent products; the aqueous products at 12 and 24 hr were 9% and 20% as fluorescent. In reaction systems that contained ethyl arachidonate, phenylalanine and phosphatidyl ethanolamine, the aqueous fluorescence was approximately one-half as great as the lipid soluble fluorescence up to 12 hr, but by 24 hr they were at the same level. Very little or no fluorescence developed in the simple peroxidizing systems of ethyl arachidonate or methyl docosahexaenoate, or in the inert phosphatidyl ethanolamine. The linear regression correlation coefficients in Table II show the excellent correlations of production of both aqueous and lipid soluble fluorescent chromophores with oxygen absorption, in most systems for up to 24 hr of oxidation. Fluorescence was fairly well correlated with TBA reactants for 6-12 hr; however the correlations generally were not as significant as the correlations with oxygen absorption.

DISCUSSION

Dipalmityl phosphatidyl ethanolamine decreased the induction period of oxidizing arachidonate and docosahexaenoate in phosphate buffer that contained ascorbate and iron; the effect of phenylalanine was variable. The induction period is influenced by factors such as oxygen pressure (8), antioxidants (9), temperature and catalysts (10). Autoxidation is usually accelerated by primary amines (11), and soybean phosphatidyl ethanolamine and choline

shorten the induction period of arachidonic acid (12). Amino acids are prooxidant to different degrees, and phenylalanine is one of the effective amino acid prooxidants (13). The oxidation of linoleate esters is increased more than 60-fold by the addition of both histidine and ionic iron, but the effect is retarded by acetate and completely repressed by phosphate (14). In the studies described here, phosphatidyl ethanolamine increased the total oxygen absorption by arachidonate and methyl docosahexaenoate, but did not absorb oxygen itself. This increase is partly due to the emulsifying property of the phospholipid. Phenylalanine shortened the induction period of arachidonic acid but did not affect the total amount of oxygen absorbed. The effect of phenylalanine on docosahexaenoate was to lengthen the induction period and to decrease the total amount of oxygen absorbed by 24 hr. The prooxidant and antioxidant effects of phenylalanine on these two fatty acids cannot be explained on the basis of these experiments. Phosphatidyl ethanolamine increased the production of TBA reactants during oxidation of arachidonate and docosahexaenoate. TBA reactants in oxidizing arachidonate systems were increased by phenylalanine; in oxidizing docosahexaenoate they were decreased. Although these differences occurred, the TBA reactant levels were well correlated with oxygen absorption until the rate of oxygen absorption decreased appreciably. The TBA reactant levels then decreased. Similar observations were made by Tarladgis and Watts (15) and by Corliss and Dugan (12).

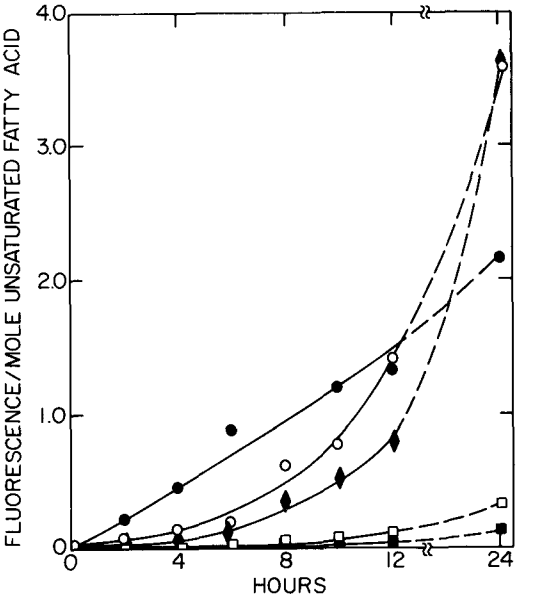


FIG. 4. Formation of water soluble fluorescent products as a function of time. Extraction procedures are described in the text. Fluorescence is expressed as mmoles equivalent glycine-1-amino-3-iminopropene fluorescence. Fluorescence excitation and emission maxima at 10 hr are given. (●) Arachidonic acid and phenylalanine, 360 nm, 440 nm; (■) methyl docosahexaenoate, 370 nm, 450 nm; (□) methyl docosahexaenoate and phosphatidyl ethanolamine, 360 nm, 455 nm; (○) methyl docosahexaenoate and phenylalanine, 360 nm, 435 nm; (◆) ethyl arachidonate, phosphatidyl ethanolamine and phenylalanine, 355 nm, 435 nm; neither phosphatidyl ethanolamine nor ethyl arachidonate were fluorescent by 24 hr.

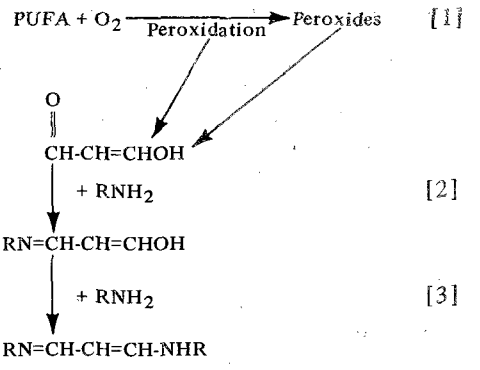
Both aqueous (1) and lipid soluble (2) fluorescent chromophores from peroxidizing animal tissue fractions have been studied previously. The aqueous pigments are of the type synthesized and characterized by Chio and Tappel (3) from amino acids and malonaldehyde. Three lipid soluble standard compounds have been prepared in this laboratory by V. Malshet. The fluorescence excitation and emission maxima, respectively, of these compounds are N-1-benzal-2,2'-hydroxyaminoethane, 362 nm, 446 nm; N-alanyl-2-hydroxynaphthlylidine, 385 nm, 436 nm; and N,N-dihexanyl-1-amino-3-iminopropene, 409 nm, 470 nm. These are similar to the range of excitation and emission maxima of the fluorochromes reported here and of those produced by reaction of malonaldehyde and dipalmityl phosphatidyl ethanolamine (360 nm excitation and 430 nm emission) (16). The characteristics of these fluorochromes are similar to those in lipid extracts of human neuronal lipofuscin (375 nm excitation and 440-460 nm emission) (17), and of human heart (365 nm excitation and 450-470 nm emission) (18).

The lipid soluble fluorescent pigments from tissues of animals stressed with high dietary unsaturated lipids in the absence of vitamin E have been used as a marker of in vivo lipid peroxidation (4,5); ceroid age pigments are a contributing source of fluorescent material. Since there are many fluorescent compounds in the body, some of which are lipids, a survey of various lipids was made to determine whether they could possibly interfere with the fluorescence of the conjugated Schiff-base products derived from reaction of carbonyls with amines. Out of 18 lipids—including triglycerides, phospholipids, cholesterol and its esters, fatty acids and retinol—assayed for fluorescence, only purified phosphatidyl ethanolamine and sheep brain cephalin had significant fluorescence; their fluorescence, as would be expected from these model studies, was characteristic of conjugated Schiff base fluorescence. Of the fluorescent lipids extracted from animal tissues by chloro-

form-methanol, only retinol has a molar extinction of fluorescence great enough to interfere with the fluorescence related to lipid peroxidation. In chloroform solution, retinol can be easily photolyzed by short term exposure to UV light, thereby removing the interference (4). From these observations it appears unlikely that other lipids interfere with fluorescence of animal extracts.

We were interested in the determination of the relative amounts of aqueous and lipid soluble fluorescence that developed during oxidation of an unsaturated fatty acid in the presence of an amino acid or aminophospholipid, or both. By 24 hr of oxidation there were low levels of aqueous fluorescent materials in the fatty acid-phosphatidyl ethanolamine reaction systems and low levels of lipid soluble fluorescence in the fatty acid-phenylalanine reaction systems. A small part of this fluorescence can be explained by carryover to the water layer by methanol of lipid soluble products and to incomplete washing of the chloroform layer to remove aqueous products. It is interesting that, in a reaction containing both phosphatidyl ethanolamine and phenylalanine, for 12 hr the rate of formation of lipid soluble fluorescent chromophores was greater than for the water soluble product. Malonaldehyde is a water soluble compound, and we had expected the rate of its reaction with a water soluble amino acid to be greater than its reaction with the lipid soluble phosphatidyl ethanolamine.

The fluorescent products that formed during oxidation of arachidonate and docosahexaenoate with phenylalanine or phosphatidyl ethanolamine were yellow. The synthetic 1-amino-3-iminopropene standards, both aqueous and lipid soluble, are yellow chromophores. Corliss and Dugan (12) noted that free amino groups significantly decreased during oxidation of phospholipids and that a brownish-yellow color developed, which they attributed to a Maillard-type browning reaction. Lea (19) reported earlier that free amino groups of phosphatidyl ethanolamine disappeared during oxidation approximately in proportion to the amount of oxygen absorbed. Both these observations probably can be attributed to the reaction of the amine group with carbonyls, mainly malonaldehyde, produced by lipid oxidation, to form Schiff base products, some of which are measured by the fluorescence technique. A conjugated Schiff base structure is required for fluorescence (3); hence monomer amine-malonaldehyde products are not fluorescent, but low levels of the dimer products are easily detected by sensitive fluorescence techniques. As indicated in the following scheme, oxygen absorbed is a measure of total peroxidation:



Throughout the reaction periods the malonaldehyde derived from peroxides (20) and intermediates of peroxidation (K. Ingold, personal communication) is probably proportional to total peroxidation, as shown in reaction [1]. The measured malonaldehyde is equal to the amount produced by peroxidation less that which has reacted [2]. A major reaction pathway is through reaction with amines, which were in excess when present in these experiments. The major products of the malonaldehyde-amine 1:1 reaction should also be proportional to the concentration of malonaldehyde and to the time it has to react with the amine. Reactions of malonaldehyde with amine and other nonamine reactions account for the maxima and subsequent decline in malonaldehyde, as shown in Table I. Fluorescent products develop by subsequent reaction of malonaldehyde-amine products with another amine [3]. Through this series of reactions the small amounts of fluorescent products are formed in amounts proportional to the overall peroxidation reaction (Table II). Although the production of fluorescent compounds was highly correlated with oxygen absorption and malonaldehyde measurements, the ratios of oxygen absorbed-malonaldehyde produced-fluorescent products in the various oxidizing systems was quite variable. The correlation between TBA reactants and fluorescence development was less significant than that between oxygen absorption and fluorescent products. This can be partly explained by the breakdown of malonaldehyde with time and by the removal of small amounts by reaction with amines, as shown in the above scheme.

ACKNOWLEDGMENT

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REFERENCES

1. Chio, K.S., U. Reiss, B. Fletcher and A.L. Tappel, Science 166:1535 (1969).
2. Dillard, C.J., and A.L. Tappel, Lipids 6:715 (1971).
3. Chio, K.S., and A.L. Tappel, Biochemistry 8:2821 (1969).
4. Tappel, A.L., in "Pathological Aspects of Cell Membranes," Vol. 1, Edited by B.F. Trump and A. Arstila, Academic Press, New York, In press.
5. Tappel, A.L., Ann. N.Y. Acad. Sci. 203:12 (1972).
6. Porta, E.A., and W.S. Hartroft, in "Pigments in Pathology," Edited by M. Wolman, Academic Press, New York, 1969, p. 191.
7. Wills, E.D., Biochem. J. 99:667 (1966).
8. Marcuse, R., and P.-O. Fredriksson, JAOCS 45:400 (1968).
9. Marcuse, R., and P.-O. Fredriksson, Ibid. 46:262 (1969).
10. Hess, P.S., and G.A. O'Hare, Ind. Eng. Chem. 42:1424 (1950).
11. Tsai, L.-S., and L.M. Smith, Lipids 6:196 (1971).
12. Corliss, G.A., and L.R. Dugan, Jr., Ibid. 5:846 (1970).
13. Marcuse, R., Fette, Seifen, Anstrichm. 63:547 (1961).
14. Saunders, D.H., J.E. Coleman, J.W. Hampson, P.A. Wills and R.W. Riemanschneider, JAOCS 39:434 (1962).
15. Tarladgis, B.G., and B.M. Watts, Ibid. 37:403 (1960).
16. Bidlack, W.R., Ph.D. thesis, University of California, Davis, 1972.
17. Hydén, H., and B. Lindström, Discus. Faraday Soc. 9:436 (1950).
18. Hendley, D.D., A.S. Mildvan, M.C. Reporter and B.L. Strehler, J. Gerontol. 18:144 (1963).
19. Lea, C.H., in "Biochemical Problems of Lipids," Edited by G. Popjak and E. LeBreton, Interscience Publishers Inc., New York, 1956, p. 81.
20. Keeney, M., in "Symposium on Foods: Lipids and their Oxidation," Edited by H.W. Schultz, Avi Publishing Co., Inc., Westport, Conn., 1962, p. 79.

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Tritium Labeling of Lipids[1]

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ABSTRACT

The elucidation of metabolic pathways, chemical reactions and biosyntheses has been greatly enhanced by the use of radioisotopes. Tritium has become an important tool in lipid research. Lipids have been labeled with tritium by a variety of techniques, including chemical synthesis, catalytic reductions, exchange reactions and radiation-induced reactions. Specific examples of labeling procedures are presented. Recent developments in tritium analysis instrumentation and procedures are also presented. Current applications of tritium as a tracer are in biological investigations and catalytic hydrogenation of lipids.

INTRODUCTION

The use of tritium as a tracer in lipid research has greatly expanded since the discovery by Wilzbach (1) that organic compounds could be labeled by a simple gas exposure technique. The concurrent development of liquid scintillation counting techniques (2,3) for detecting this low β-energy emitting isotope was another important factor leading to its increased application. Subsequent investigations have revealed the limitations of the gas exposure technique; however the advantages of using this isotope have led to the development of a variety of alternate methods of producing labeled compounds.

Since tritium is a hydrogen isotope, radioactive labeling is often accomplished easier and quicker than with other radioisotopes (exchange reactions, reductions, etc.). Extremely high specific activities can be achieved, and rather large quantities may be used, at a much lower cost than other isotopes. The cost of the raw materials, either ^{14}C-carbonate or tritium gas, shows a difference of \$6.00/mCi and %0.002-\$0.003/mCi, respectively. Comparatively, carbon 14 has a half-life 460 times as long (^{14}C, 5760 years; ^{3}H, 12.26 years) and a β energy 8.6 times as great (^{14}C, 0.049 Mev; ^{3}H, 0.0057 Mev).

In presenting some of the labeling techniques developed, the specific use, where applicable, of the labeled lipid produced will be described. Some recent developments in analysis instrumentation will also be point out.

RADIATION-INDUCED REACTIONS

As stated, the Wilzbach gas exposure technique (1) was an important development in the history of tritium as a tracer. The technique involves the exposure of the compound to be labeled to curie amounts of tritium gas for several days. In practice, a lipid to be labeled is contained in a sealed vial along with the tritium gas. The vial is rotated to produce a thin film surface to the gas, and exposure is continued from 4 to 18 days.

Nystrom et al. (4) reported the first attempt to label lipid materials by gas exposure. They irradiated gram amounts of saturated methyl esters with 1 Ci and 5 Ci tritium sources. Their data indicate that the tritium incorporated was roughly related to the time of exposure and intensity of the source.

While substitution of hydrogen by tritium took place as anticipated with the saturated methyl ester, labeling unsaturated methyl esters involved more complicated reactions (5). Subsequent analysis of the tritiated methyl esters by oxidative cleavage of the double bonds according to the method of Jones and Stolp (6) showed that labeling occurred through addition to the double bond.

Methyl stearolate was exposed to a 2.6 Ci of tritium for 20 days. Tritium incorporation was 210.0 mCi, of which 79.6% was recovered as the acid (7). The major product formed during exposure of methyl stearolate was 9,10-tritiooctadecenoate, both the *cis* and *trans* isomer. Small amounts of radioactive methyl stearate as a result of saturation and methyl stearolate arising from substitution were also formed.

When radiochemical products are separated by chromatography (thin layer, liquid columns, preparative gas liquid chromatography) from their inactive isologous parent, fatty acids of extremely high specific activities are isolated. Development of argentation chromatography (8) provided a simple technique to separate *cis*- and *trans*-9,10-octadecenoates formed by addition to methyl stearolate.

CHEMICAL SYNTHESIS

Both Sgoutas and Kummerow (9) and

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

[2]ARS, USDA.

TABLE I

Synthesis of Tritiated Polyunsaturated Fatty Acid Methyl Esters[a]

Starting acetylenic compound[b]	Tritiating agent[c]	Yield,[c] %		Specific activity, mCi
		Chemical	Radiochemical	
$CH_3(CH_2)_4C{\equiv}CCH_2C{\equiv}C(CH_2)_7COOCH_3$	$NaBH_4(^3H)$	82.3	27.8	9.25
$CH_3(CH_2)_3C{\equiv}CCH_2C{\equiv}C(CH_2)_8COOCH_3$	$NaBH_4(^3H)$	78.6	26.9	8.80
$CH_3(CH_2)_3C{\equiv}CCH_2C{\equiv}C(CH_2)_8COOCH_3$	$CH_3COOH(^3H)$	82.8	---	1.92
$CH_3(CH_2)_3C{\equiv}CCH_2C{\equiv}C(CH_2)_8COOCH_3$	$NaBH_4(^3H)$	72.6	25.7	26.20
trans $CH_3(CH_2)_4CH{=}CHCH_2C{\equiv}C(CH_2)_7COOCH_3$	$NaBH_4(^3H)$	85.2	29.0	11.60
trans $CH_3(CH_2)_5CH{=}CHCH_2C{\equiv}C(CH_2)_7COOCH_3$	$NaBH_4(^3H)$	81.7	28.5	11.40
$CH_3(CH_2)_4C{\equiv}CCH_2C{\equiv}CCH_2C{\equiv}C(CH_2)_4COOCH_3$	$NaBH_4(^3H)$	70.7	26.0	30.12
$CH_3(CH_2)_4C{\equiv}CCH_2C{\equiv}CCH_2C{\equiv}C(CH_2)_4COOCH_3$	$CH_3COOH(^3H)$	75.4	---	2.52

[a]Source: Sgoutas et al. (14).

[b]Ca. 1 mmol of each.

[c]Specific activity: $NaBH_4(^3H)$, 20-50 mCi/mmol; $CH_3COOH(^3H)$, 1.2 mCi/mmol. Chemical yield based on starting methyl ester. Radiochemical based on $NaBH_4(^3H)$.

Stoffel (10) report labeling of polyunsaturated fatty acids by half-hydrogenation of polyacetylenic acid using Lindlar catalyst in a tritium atmosphere. They (9) describe the total synthesis of octadeca-9,12-diynoic acid and its reduction to almost pure octadeca-9,12-dienoic acid with a *cis* configuration. Stoffel emphasizes that the technique is applicable to any polyunsaturated acid for which the isologous polyacetylenic acid would be prepared. Oxidative degradation indicates that greater than 95% of the tritium radioactivity was at the double bond positions.

Stoffel (10) further demonstrated the total synthesis of linoleic,-γ-linolenic, eicosa-11,14-dienoic and eicosa-8, 11,14-trienoic acid, each labeled with tritium in the terminal methyl group. According to his scheme, tritium is introduced early in the synthesis by catalytic hydrogenation of pentyn-1-ol-5 in ethyl acetate with tritium gas over platinum oxide to yield tritiated *n*-amylalchol. The specific acitivity was 2.3 μCi/μmol. The polyunsaturated product of the synthesis scheme was shown to be better than 95% pure, and the specific acitivity was 2.3 μCi/μmol. The identical specific activities of the starting labeled compound and the synthesized product demonstrated the stability of the label to chemical manipulation.

Tenny et al. (11) had earlier prepared *cis*-9,10-octadecenoic acid by reducing stearolic acid with tritium gas in the presence of 5% palladium on charcoal. The product showed a specificity of greater than 90% of the activity at the 9 and 10 positions. Specific radioactivity of the oleic acid was more than 300 mCi/mM. In their experiments, because uptake of hydrogen did not cease after the double bond had formed, the quantity of hydrogen had to be limited and the reaction period had to be short to prevent formation of stearic acid. Mounts et al. (12) labeled *cis*-9,10-octadecenoate by reducing stearolate with a copper-chromite catalyst. The catalyst was stirred with a small amount of tritiated water and hydrogen gas at atmospheric pressure for 1 hr at room temperature. After raising the temperature to 170 C, methyl stearolate was injected into the closed system, and reduction was continued until there was no further uptake of hydrogen. As reported by Koritala (13), the reduction of stearolate with copper-chromite catalysts is stereospecific, and no methyl stearate is produced. The specific activity of the product was 5.27 MCi/mM, and more than 95% of the tritium was located at the double bond position. This use of tritiated water as the source of the tritium in the reduction was important as previous catalytic reductions required the presence of tritium gas.

Noncatalytic reductions of acetylene bonds have also been used to introduce tritium into lipid materials. Sgoutas et al. (14) used disiamylborane, prepared in situ, to reduce polyacetylenic esters. As shown in Table I, sodium borotritide or tritiated acetic acid was used to prepare several polyunsaturated fatty acids. They noted that a contaminant was eluted together with the methyl ester from a silicic acid column. Final purification was achieved by preparative gas liquid chromatography.

Diimide reduction of unsaturated bonds has been reported by Koch (15). He described two methods for obtaining the labeled diimide; in each, tritiated water was the source of the label. In one method potassium azodicarboxylate was

TABLE II

Specific Activities of Degradation Products in Percentage of Value for Stearic Acid[a]

Chain length of degraded acids	Theoretical specific activity for 9,10-^{3}H-stearic acid	Stearic acid from diimide reduction
18	100	100
17	100	98
16	100	99
15	100	100
14	100	99
13	100	101
12	100	96
11	100	97.5
10	50	48.5
9	0	0
8		
7		
6		
5		

[a]Source: Koch (15).

decomposed with labeled hydrogen ions in a pyridine medium; in the other, the labeled diimide is obtained by exchange labeling and oxidation of hydrazine in acetonitrile. Unsaturated esters were reduced on a 10-100 mg scale, and molar specific activities ranged from 8 to 20% of the original tritiated water. Koch found that the reaction involving the potassium azodicarboxylate was enhanced by increasing the temperature to 100 C and that for quantitative conversion this reaction was preferred over one involving hydrazine. Results obtained by oxidative degradation ($KMNO_4$ with refluxing acetone) of stearic acid produced by reduction of oleic acid are given in Table II. These results agree with specific labeling at the 9,10 position. The diimide reaction with subsequent oxidative degradation of the tritiated saturated product was proposed by Koch as a method to determine the structure of polyunsaturated acids.

Another novel application of the products of diimide reduction was developed by Dutton (unpublished). Linolenic acid was reduced with labeled diimide. The tritiated products served as an internal standard during the ozonolysis-pyrolysis analysis (16) of products of catalytic hydrogenation. Aldehyde and aldehyde ester fragments of the inactive unknown were identified by correlating them with the radioactive fragments from the tritiated products.

Labeling with tritium at the double bond has proved to be quite satisfactory for most biological and chemical investigations. However, while investigating the kinetics of hydrogenation of polyunsaturated fats (17), the location of tritium at the active site of reaction, the double bond, proved unsatisfactory. Mounts and Dutton (18) developed a high-vacuum technique to label esters with tritium in the methyl group. Purity of the radiochemical product was identical to the purity of the inactive acid used in the reaction. This reaction has provided a simple technique for preparing labeled methyl esters of unusual fatty acids including *cis*- and *trans*-octadecenoates with double bond positions from $\Delta 3$ to $\Delta 16$ (17), conjugated octadecadienoates and α- and β-eleostearates. This type of labeled compound can be used to good advantage in those applications where the integrity of the methyl ester is maintained.

ANALYSIS INSTRUMENTATION

In 1961, Dutton (19) described the serial collection of the effluent from gas liquid chromatography columns for scintillation counting. This technique gave the highest sensitivity for compounds of low specific acitivity; i.e., 0.5-100 nCi/mg. In 1969, Thomas and Dutton (20) significantly improved the high resolution of the system when they applied modern data-processing techniques using a high speed digital computer. By combining and averaging from two to four repeated analyses on the same sample, a true differential curve of the distribution of radioactivity in the column effluent was approximated. The automatic collection system was modified from that originally described. Important features of the collection system include: (a) a high temperature maintained in the gas stream up to the point of its contact with the scintillation solvent, (b) holdup volume at all points of the liquid system kept to a minimum to eliminate peak tailing and (c) joining the sample and reference streams from the exit of the thermal

conductivity cells, thus eliminating ca. 80% of the noise introduced by gas pressure changes within the cell. This noise was further reduced by an air reservoir at the pump.

Clifford et al. (21) chose a flow-through cell for the continuous monitoring of effluents from liquid chromatographic columns. Scintillators were lithium-cerium glass, insoluble in all solvents except hydrofluoric acid, and europium-activated calcium fluoride powder, soluble only in ammonia. The efficiency for ^{3}H in solution was reported as less than 1%, whereas ^{14}C efficiencies were 20 and 50%. Assays at the Northern Lab. with the europium-activated calcium fluoride scintillator confirm a tritium efficiency of 0.3%. This accessory has been used to detect tritium during column chromatography for purifying synthesized labeled lipids.

RECENT APPLICATIONS

At present, applications of tritium-labeled lipids at the Northern Lab. are to studies of kinetics of catalytic hydrogenation of fats and to the metabolism of isomerized fats. For example, methoxyl tritium-labeled methyl esters of unusual fatty acids have been used to determine competitive hydrogenation rates in liquid-phase reactions (18). The hydrogenation products were analyzed with a gas liquid radio chromatographic system (22).

During the catalytic hydrogenation of polyunsaturated oils, a variety of geometric and positional isomers are formed. Isomeric octadecenoates labeled with tritium at the double bond have been fed to laying hens in double label experiments with ^{14}C-labeled *cis*-9,10-octadecenoate to determine the comparative metabolism of natural and unnatural isomers (12). The egg served as a "biological trap" giving an automatic daily biopsy with which to study the metabolism of the isomers in the hen. Dual label analysis of the egg lipids was performed with a three-channel liquid scintillation counter designed to give minimum overlap with maximum efficiency of counting.

REFERENCES

1. Wilzbach, K.E., J. Amer. Chem. Soc. 79:1013 (1957).
2. Reynolds, G.T., F.B. Harrison and G. Salvini, Phys. Rev. 78:488 (1950).
3. Kallmann, H., and M. Furst, Ibid. 79:857 (1950).
4. Nystrom, R.F., L.H. Mason, E.P. Jones and H.J. Dutton, JAOCS 36:212 (1959).
5. Jones, E.P., L.H. Mason, H.J. Dutton and R.F. Nystrom, J. Org. Chem. 25:1413 (1960).
6. Jones, E.P., and J.A. Stolp, JAOCS 35:71 (1958).
7. Dutton, H.J., E.P. Jones, V.L. Davison and R.F. Nystrom, J. Org. Chem. 27:2648 (1962).
8. Emken, E.A., C.R. Scholfield and H.J. Dutton, JAOCS 41:388 (1964).
9. Sgoutas, D.S., and F.A. Kummerow, Biochemistry 3:406 (1964).
10. Stoffel, W., JAOCS 42:583 (1965).
11. Tenny, K.S., S.C. Gupta, R.F. Nystrom and F.A. Kummerow, Ibid. 40:172 (1963).
12. Mounts, T.L., E.A. Emken, W.K. Rohwedder and H.J. Dutton, JAOCS 48:99A (Abstr. 125) (1971).
13. Koritala, S., Ibid. 45:708 (1968).
14. Sgoutas, D.S., H. Sanders and E.M. Yang, J. Lipid Res. 10:642 (1969).
15. Koch, G.K., J. Label. Compounds 5:110 (1969).
16. Bitner, E.D., V.L. Davison and H.J. Dutton, JAOCS 46:113 (1969).
17. Scholfield, C.R., T.L. Mounts, R.O. Butterfield and H.J. Dutton, Ibid. 48:237 (1971).
18. Mounts, T.L., and H.J. Dutton, J. Label. Compounds 3:343 (1967).
19. Dutton, H.J., JAOCS 38:631 (1961).
20. Thomas, P.J., and H.J. Dutton, Anal. Chem. 41:657 (1969).
21. Clifford, K.H., A.J.W. Hewett and G. Popjak, J. Chromatogr. 40:377 (1969).
22. Nelson, D.C., P.C. Rissler, Jr., and R.C. Hawes, Anal. Chem. 35:1575 (1963).

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Fluorescent Products of Lipid Peroxidation: I. Structural Requirement for Fluorescence in Conjugated Schiff Bases

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ABSTRACT

The structural requirement for fluorescence in Schiff bases was defined. Aldehydes and amines were reacted and the structures of the Schiff bases N-hydroxyethyl-1-imino-2′,4′-hexadiene (I), N-1-benzal-2-hydroxyaminoethane (II), and N-1-benzal-2,2′-hydroxyaminoethane (III) were established by elemental analysis, IR spectral analysis and mass spectral analysis. The structures of N-alanyl-2-hydroxy-naphthylidine (IV) and N,N′-dileucinyl-1-amino-3-iminopropene (V) had been established previously. III, IV and V were fluorescent compounds and I and II were not. The results of these analyses suggest that an electron donating group in conjugation with an imine is the structure required for fluorescence.

INTRODUCTION

Oxidation of polyunsaturated fatty acids produces malonaldehyde and numerous other carbonyls (1). Baker and Wilson (2) showed that UV irradiation of autoxidized unsaturated fatty acids produces carbonyl products. Crawford et al. (3) showed that the mechanism by which glycine reacts with malonaldehyde involves 1,4 addition to the α,β-unsaturated carbonyl system of malonaldehyde to form the enamine, N-prop-2-enolaminoacetic acid. Chio and Tappel (4) showed that the crosslinking reaction of malonaldehyde with amino acids produces fluorescent chromophores with emission maxima at 460-470 nm when excited at 360-400 nm. The fluorescence of these com-

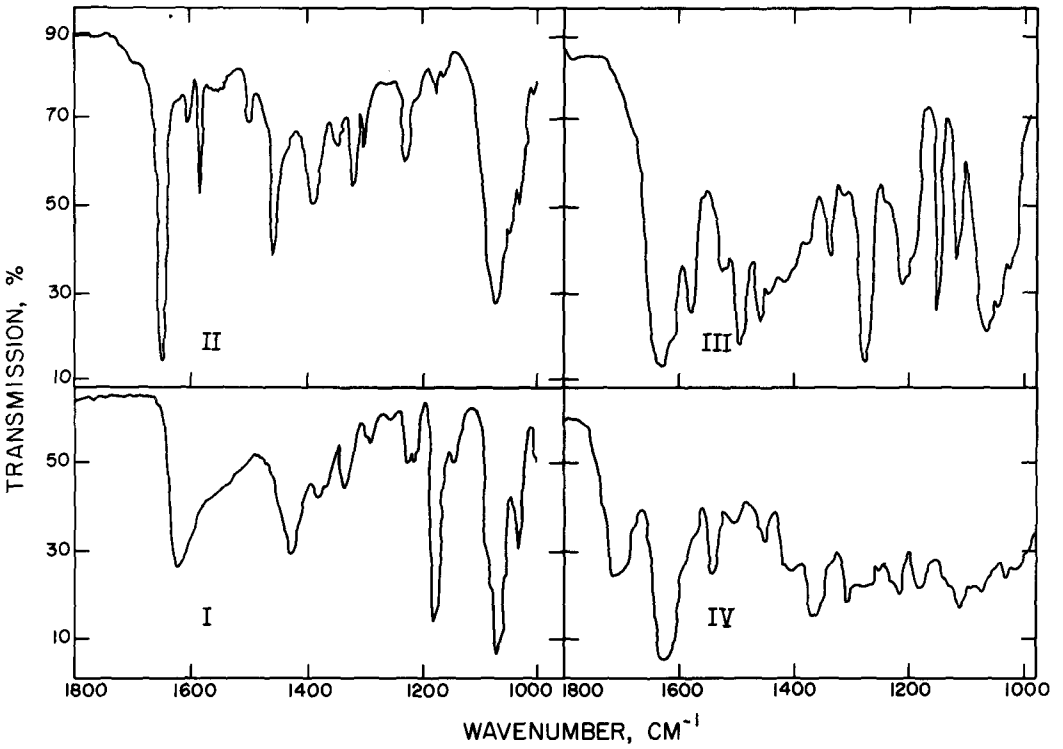


FIG. 1. IR spectra of (II) N-1-benzal-2-hydroxyaminoethane, (I) N-2-hydroxyethyl-1-imino-2′,4′-hexadiene, (III) N-1-benzal-2,2′-hydroxyaminoethane and (IV) N-alanyl-2-hydroxy-naphthylidine.

pounds is due to a surprisingly simple fluorochrome structure, the 1-amino-3-imino unsaturated system, R-N=C-C=C-N-R.

The formation of compounds with similar fluorescence spectral characteristics results from lipid peroxidation of membranes (5). Similar fluorescent products are formed in vivo, as evidenced by the accumulation of fluorescent ceroid and lipofuscin pigments in various tissues (6,7).

In addition to malonaldehyde some other unsaturated aldehydes, such as 2,4-hexadiene-1-al, are produced in vitro and in vivo by lipid peroxidation. The mechanism for their production has been proposed by Lillard and Day (8). It needs to be determined whether or not these aldehydes when reacted with free amino groups, as in proteins and in phospholipids, would form fluorescent chromophores. This study reports on the synthesis and properties of conjugated Schiff bases and the molecular structures required for fluorescence.

EXPERIMENTAL PROCEDURES

Materials

2,4-Hexadiene-1-al was obtained from K and K Labs., Inc., salicylaldehyde and 2-hydroxynaphthaldehyde from Eastman Organic Chemicals, and ethanolamine from Aldrich Chemical Co., Inc.

Methods

Preparation of N-2-Hydroxyethyl-1-imino-2,4-hexadiene (I): Ethanolamine (0.01 mol) was dissolved in 200 ml anhydrous ethyl alcohol. 2,4-Hexadiene-1-al (0.01 mol) was added. The mixture was stirred for 12 hr under anhydrous conditions. Solvent was removed under reduced pressure. The brown-colored product was dissolved in 300 ml ethyl ether and filtered. The filtrate was evaporated and the residue dissolved in a minimal amount of ethyl acetate, filtered through cotton and stored at -18 C for 6 hr. The crystals formed were dissolved and recrystallized from ethyl acetate, which gave a 65% yield of a white solid with a melting point of 73 C.

Analysis. Calculated for $C_8H_{13}N_1O_1$: C, 69.06; H, 9.35; N, 10.67. Found: C, 68.88; H, 9.17; N, 10.71.

Preparation of N-1-benzal-2-hydroxyaminoethane (II): Ethanolamine (0.01 mol) was dissolved in 20 ml anhydrous ethyl alcohol. Benzaldehyde (0.01 mol) was added and the reaction mixture was stirred for 6 hr under anhydrous conditions. Solvent was removed under reduced pressure. The yellow, oily product obtained was further purified by distillation

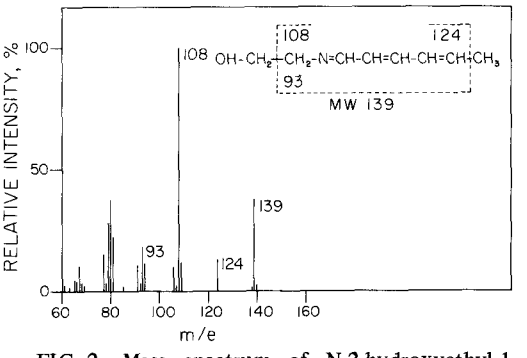


FIG. 2. Mass spectrum of N-2-hydroxyethyl-1-imino-2',4'-hexadiene (I).

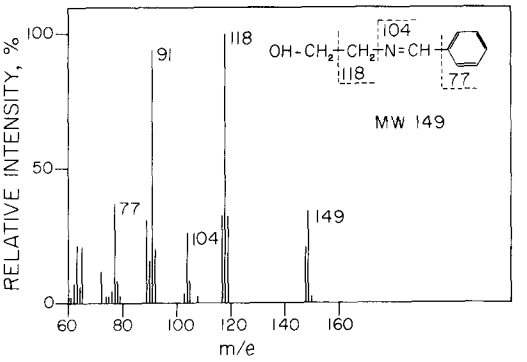


FIG. 3. Mass spectrum of N-1-benzal-2-hydroxyaminoethane (II).

at 85-86 C/0.05 mm. The colorless oil was obtained in 80% yield.

Analysis. Calculated for $C_9H_{11}N_1O_1$: C, 72.48; H, 7.38; N, 9.40. Found: C, 72.17; H, 7.34; N, 9.56.

Preparation of N-1-benzal-2,2'-hydroxyaminoethane (III): Ethanolamine (0.01 mol) was dissolved in 20 ml anhydrous ethyl alcohol. Salicylaldehyde (0.01 mol) was added, and the reaction mixture was stirred for 6 hr under anhydrous conditions. Solvent was removed under reduced pressure. The orange-colored oil produced was distilled at 105-106 C/0.05 mm. The final product was a yellow oil obtained in 79% yield.

Analysis. Calculated for $C_9H_{11}N_1O_2$: C, 65.45; H, 6.67; N, 8.48. Found: C, 65.19; H, 6.49; N, 8.36.

Preparation of N-alanyl-2-hydroxy-naphthylidine (IV): This compound was prepared by the procedure described by McIntire (9).

N,N'-Dileucinyl-1-amino-3-iminopropene (V): This compound was prepared as described by Chio and Tappel (4).

Absorption Spectroscopy

The electronic absorption spectra in the

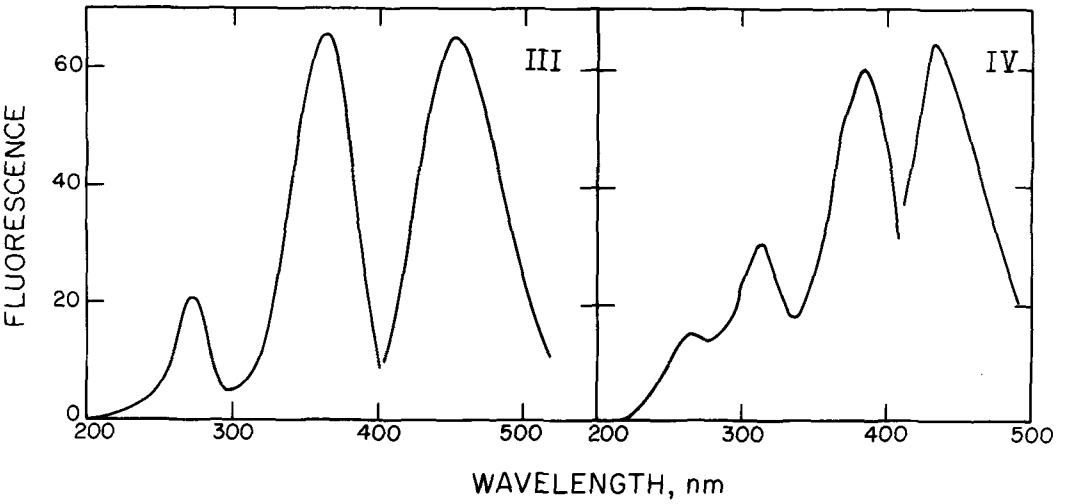


FIG. 4. Excitation and fluorescence spectra of (III) N-1-benzal-2,2'-hydroxyaminoethane with excitation at 362 nm and emission at 430 nm; and of (IV) N-alanyl-2-hydroxynaphthylidine with excitation at 385 nm and emission at 436 nm. The meter multiplier setting was 0.01. Other instrumentation parameters are described in the text.

visible and UV regions were measured at room temperature with a Beckman DB-G spectrophotometer.

IR Spectroscopy

IR spectra of the compounds were obtained with a Perkin-Elmer 457 IR spectrophotometer. KBr windows of solid products were prepared by grinding 100 mg oven-dried spectrograde KBr with 1 mg of the product. A single drop of the liquid compounds was spread between two NaCl discs (each 25.5 x 5 mm).

Mass Spectroscopy

Mass spectra were determined on a Varian M66 mass spectrometer operating at an ionizing potential of 70 ev with a 3 μamp electron current. Samples were introduced through a solid probe at 30 C.

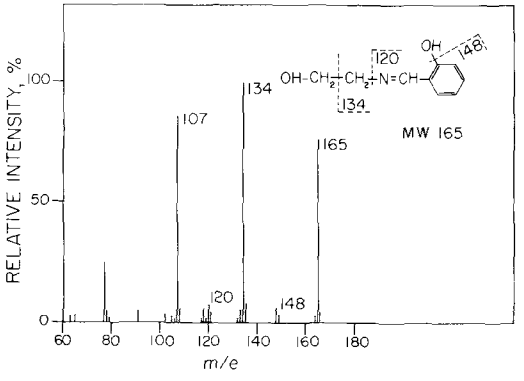


FIG. 5. Mass spectrum of N-1-benzal-2,2'-hydroxyaminoethane (III).

Measurement of Fluorescence

Fluorescence and excitation spectra were determined with an Amico-Bowman Spectrophotofluorometer (American Instrument Co., Inc.) calibrated with quinine sulfate. The slit arrangement for recording fluorescence spectra was slits 3, 4 and 6, set at 3, 1 and 3 mm, respectively. The sensitivity setting was 50. Spectra were recorded on an X-Y recorder (Houston Instrument). Under these instrument parameters, 1 μg quinine sulfate per milliliter 0.1 N H_2SO_4 had a fluorescence intensity of 60 at a 0.3 meter multiplier setting.

RESULTS

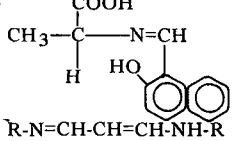
N-2-Hydroxyethyl-1-imino-2',4'-hexadiene (I)

Elemental analysis of the synthesized N-2-hydroxyethyl-1-imino-2',4'-hexadiene agreed with its predicted composition. The IR spectra showed characteristic broad stretching at 1625 cm^{-1} (Fig. 1) due to an imine in conjugation with an olefin. The UV spectra indicated a major absorption peak at 265 nm (ϵ_m = 33,757). This compound was not fluorescent.

The mass spectrum showed a parent peak at m/e 139 with additional peaks at 124 $(M-15)^+$, m/e 108 $(M-31)^+$ and m/e 93 $(M-46)^+$ (Fig. 2). Accurate mass spectral analysis gave m/e 139.0981 in agreement with the calculated value of 139.0997 for $C_8H_{13}NO$. The base peak 108 corresponds to the loss of $-CH_2OH$

TABLE I

Structural Requirement for Fluorescence of Schiff Bases Formed from 2-β-Unsaturated Aldehydes

Reactant	Schiff base products	Electron donating group in conjugation with imine	Relative molar fluorescence[a]
Ethanolamine + 2,4,hexadiene-1-al	(I) $OH\text{-}CH_2\text{-}CH_2\text{-}N{=}CH\text{-}CH{=}CH\text{-}CH{=}CH\text{-}CH_3$	None	0
Ethanolamine + benzaldehyde	(II) $OH\text{-}CH_2\text{-}CH_2\text{-}N{=}CH$- 	None	0
Ethanolamine + salicylaldehyde	(III) $OH\text{-}CH_2\text{-}CH_2\text{-}N{=}CH$- 	-OH	4.1×10^{-3}
Alanine + 2-OH-naphthaldehyde	(IV) 	-OH	1.7×10^{-2}
Amino acids + malonaldehyde	(V) R-N=CH-CH=CH-NH-R R = amino acids or amines	-NH	2.9×10^{-1}

[a]Relative to the molar fluorescence of quinine sulfate equal to 1.00.

from the parent molecule to give an imine fragment.

N-1-Benzal-2-hydroxyaminoethane (II)

Elemental analysis of the compound agreed with the predicted composition of N-1-benzal-2-hydroxyaminoethane. The IR spectrum showed characteristic stretching at 1647 cm^{-1} (Fig. 1), indicating an imine in conjugation with the aromatic ring. The UV absorption spectrum had a major peak at 246 nm (ϵ_m = 1,746). This compound was not fluorescent.

The mass spectrum showed a parent peak at m/e 149 with additional peaks at m/e 118 $(M\text{-}31)^+$, m/e 104 $(M\text{-}45)^+$, m/e 91 of tropylium ion (M-58) and m/e 77 (M-72) (Fig. 3). Accurate mass spectral analysis gave m/e 149.0834, in agreement with the calculated value of 149.0841 for $C_9H_{11}NO$. The base peak m/e 118 corresponds to a loss of $-CH_2OH$ from the parent molecule to give an imine fragment.

N-1-Benzal-2,2'-hydroxyaminoethane (III)

Elemental analysis of the synthesized N-1-benzal-2,2'-hydroxyaminoethane agreed with its predicted composition. The IR spectrum showed a characteristic band at 1630 cm^{-1} (Fig. 1) for an imine in conjugation with the aromatic ring. The UV absorption spectrum showed one absorption band at 254 nm (ϵ_m = 11,000), one at 316 nm (ϵ_m = 3575) and one at 404 nm (ϵ_m = 1443). The fluorescence was measured in chloroform-methanol 2:1 v/v. The fluorescence spectra (Fig. 4) showed an excitation maximum at 362 nm and an emission maximum at 446 nm. The relative molar fluorescence intensity with respect to quinine sulfate was 4.1×10^{-3}.

The mass spectrum showed a parent peak at m/e 165 with additional peaks at m/e 134 $(M\text{-}31)^+$, m/e 120 $(M\text{-}45)^+$ and m/e 107 of tropylium ion (M-58) (Fig. 5). Accurate mass spectral analysis gave m/e 165.0767 in agreement with the calculated value of 165.0790 for $C_9H_{11}NO_2$. The base peak m/e 134 corresponds to a loss of $-CH_2OH$ from the parent molecule to give an imine fragment.

N-Alanyl-2-hydroxynaphthlydine (IV)

The structure of this compound was determined by elemental analysis by McIntire (9). The IR spectrum showed a characteristic band at 1625 cm^{-1}, indicating an imine in conjugation with an aromatic ring (Fig. 1). The fluorescence was measured in chloroform-methanol 2:1 v/v. The spectra showed an excitation maximum of 385 nm and an emission maximum of 436 nm (Fig. 4). The relative molar fluorescence with respect to quinine sulfate was 1.72×10^{-2}

DISCUSSION

The major aim of this research was to define the molecular structure required in Schiff bases produced by the reaction of aldehydes with amines in order for them to be fluorescent. The structures of products I-IV were established in these studies by synthesis from known reactants, elemental analysis, IR spectral analysis

and mass spectral analysis. Mass spectral analysis was among the most definitive of techniques used.

IR spectra, of all the Schiff bases prepared, showed a band at the 1620-1660 cm^{-1} region, which indicates an imine in conjugation with an olefin or an aromatic bond. These results are in agreement with those of aromatic Schiff bases prepared and studied by Margerum and Sousa (10).

Aromatic primary amines react with malonaldehyde to produce fluorescent Schiff bases, such as N,N'-disubstituted 1-amino-3-iminopropenes (11). It was previously shown (4) that Schiff base products with the general formula R-N=C-C=C-N-R are produced when malonaldehyde crosslinks with the primary amino groups of amino acids, and that these products have fluorescent spectral characteristics similar to those of products of lipid peroxidation (5) and to lipofuscin and ceroid pigments (7,8).

The chemical and structural properties of Schiff bases synthesized in this study are presented in Table I. Compounds III, IV and V are fluoroscent, and compounds I and II do not fluoresce. From the structural formula one can see that compound V has an -NH group in conjugation with the imine, while compounds III and IV have an -OH group in conjugation with the imine. Compounds I and II do not have an electron donating group in conjugation with the imine. We conclude that the structure required for fluorescence is a Schiff base in conjugation with a fairly strong electron donating group. The relatively low fluorescence of compounds III and IV correlates with the -OH group being less electron donating than the -NH- group in compound V (12). Based upon the evidence for a structural requirement for fluorescence, we also conclude that fluorescent products produced during lipid peroxidation derive mainly from malonaldehyde, that they have fluorochromic structures like V, and that the condensation of unsaturated carbonyls with amines as in compound I will not produce the structure required for fluorescence.

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REFERENCES

1. Dahl, L.K., E.G. Hill and R.T. Holman, Arch. Biochem. Biophys. 98:253 (1962).
2. Baker, N., and L. Wilson, J. Lipid Res. 7:253 (1966).
3. Crawford, D.L., T.C. Yu and R.O. Sinnhuber, J. Agr. Chem. 14:182 (1966).
4. Chio, K.S., and A.L. Tappel, Biochemistry 8:2821 (1969).
5. Dillard, C.J., and A.L. Tappel, Lipids 6:715 (1971).
6. Porta, E.A., and W.S. Hartroff, in "Pigments in Pathology," Edited by M. Wolman, Academic Press, New York, 1969, p. 191.
7. Tappel, A.L., in "Pathological Aspects of Cell Membranes," Vol. 1, Edited by B.F. Trump and A. Aristila, Academic Press, New York, In press.
8. Lillard, D.A., and E.A. Day, JAOCS 41:549 (1964).
9. McIntire, F.C., J. Amer. Chem. Soc. 69:1377 (1947).
10. Margerum, J.D., and J.A. Sousa, Appl. Spectrosc. 19:91 (1965).
11. Sawicki, E., T.W. Stanley and H. Johnson, Anal. Chem. 35:199 (1963).
12. March, J., in "Advanced Organic Chemistry: Reaction, Mechanism, and Structure," McGraw-Hill, New York, 1968, p. 496.

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Fluorescent Product Formation and Changes in Structure of DNA Reacted with Peroxidizing Arachidonic Acid

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ABSTRACT

Calf thymus DNA was reacted with peroxidizing arachidonic acid at 37 C for 76 hr. Fluorescent DNA products increased with reaction time. These products had characteristic fluorescence spectra with maximum excitation at 315 nm and maximum fluorescence at 420 nm. Structural changes occurred in the DNA reacted with peroxidizing arachidonic acid, as observed by decreased melting point, decreased hyperchromicity, partial resistance to hydrolysis by deoxyribonuclease and by decreased template activity for rat liver RNA polymerase.

INTRODUCTION

Malonaldehyde is one of the major carbonyl products formed during peroxidation of unsaturated fatty acids (1,2). Malonaldehyde reacts with amino groups, as in amino acids, to form fluorescent, conjugated Schiff base products (3) with the basic structure R-N=C-C=C-N-R. Similar fluorescent products, with maximum fluorescence emission at 460 nm and maximum excitation at 390 nm, form when ribonuclease A is reacted with malonaldehyde and with peroxidizing ethyl arachidonate (4). Malonaldehyde and glyoxal react with DNA in vitro and in vivo (5). These aldehydes react probably by crosslinking with guanine, cytidine and, to a lesser degree, adenine, all of which carry amino groups. Changes in the thermal denaturation profiles and partial resistance to degradation by deoxyribonuclease also occur. Reiss et al. (6) have shown that the reaction between malonaldehyde and DNA results in the formation of fluorescent products. A linear correlation exists between the formation of the fluorescent products and the decrease in template activity of DNA as a function of reaction time.

The present report describes the formation of fluorescent products, changes occurring in DNA structure and the effect on template activity during reaction of calf thymus DNA with peroxidizing arachidonic acid.

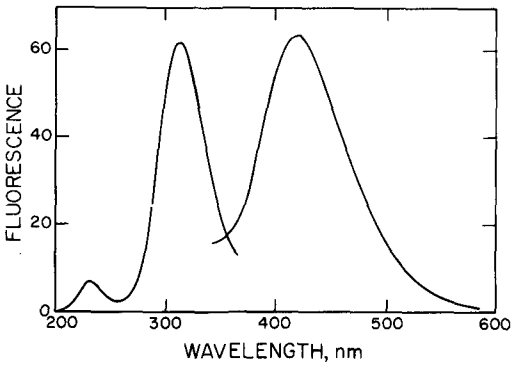

FIG. 1. Fluorescence spectra of DNA-arachidonate product. After ethanol precipitation, the samples were dialyzed against distilled water for 48 hr. DNA concentration was 0.5 mg/ml; excitation 315 nm and emission 420 nm.

MATERIALS AND METHODS

Reaction of DNA with Peroxidizing Arachidonic Acid

Arachidonic acid (Hormel Institute) was emulsified in 0.1 x SSC solution (15 mM NaCl, 1.5 mM trisodium citrate), pH 4.6, with a sonicator (Quigley-Rochester, Inc.). The emulsion was combined with a solution of calf thymus DNA (Mann Research Labs.) in 0.1 x SSC, pH 4.6. The final concentration of arachidonic acid was 0.3 M, and that of the DNA was 0.5 mg/ml. One drop of toluene was added to

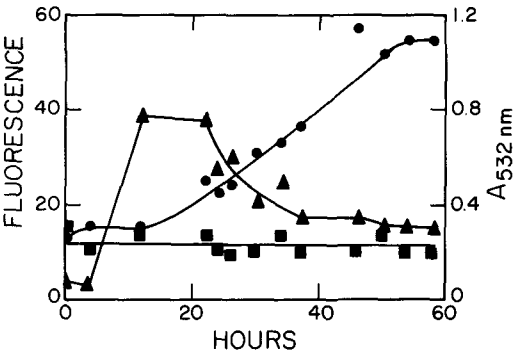

FIG. 2. Production of fluorescence and TBA reactive materials during the reaction of DNA with peroxidizing arachidonic acid. Preparation of samples is described in Materials and Methods. Fluorescence (excitation 315 nm, emission 420 nm) of DNA reacted with arachidonic acid (•) and of control DNA (■). TBA reactive materials of the supernatant portions obtained after precipitation of DNA by ethanol are expressed as $A_{532\ nm}$ (▲).

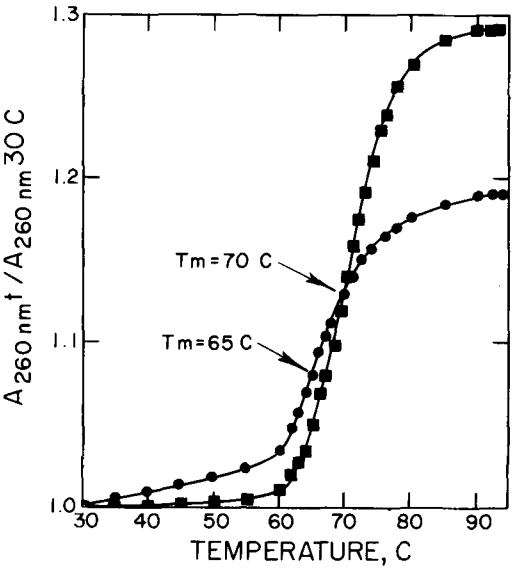

FIG. 3. Melting profiles and melting point (Tm) values of DNA-arachidonate product. The DNA concentration was adjusted to 0.4 A_{260} unit per milliliter 0.1 x SSC solution, pH 6.8. DNA-arachidonate product (•) and DNA control (▪).

prevent bacterial growth, and the mixture was reacted at 37 C with shaking. Aliquots were withdrawn at various time intervals, and the reaction was stopped by precipitating the DNA with 2 volumes 95% ethanol that contained 2% potassium acetate (-20 C). The samples were centrifuged at top speed in a clinical centrifuge. The supernatant fractions were removed and assayed for fluorescence and for 2-thiobarbituric acid (TBA) reactants. The precipitants of DNA were washed twice with 95% ethanol, dissolved in 0.1 x SSC solution, pH 6.8, and

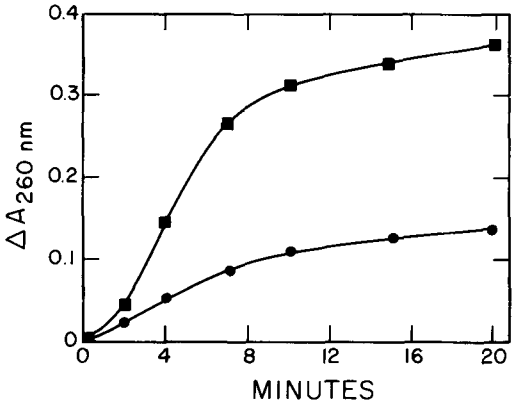

FIG. 4. Hydrolysis by deoxyribonuclease I. DNA-arachidonate product (•) and control DNA (▪). Reaction conditions are described in Materials and Methods.

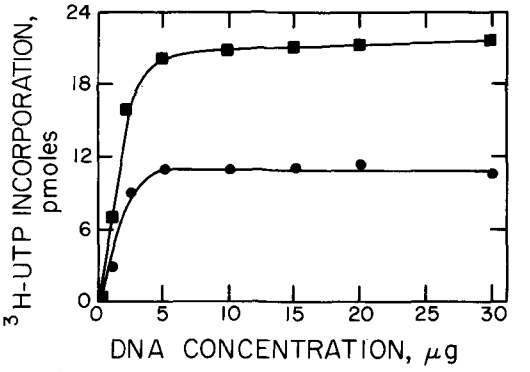

FIG. 5. Template activity for RNA polymerase. DNA-arachidonate product (•) and DNA control (▪). Assay conditions are described in Materials and Methods.

dialyzed against the same solution to remove unreacted fatty acid products. Controls of DNA without arachidonic acid and the arachidonic acid without added DNA were treated under the same conditions.

Measurements of Fluorescence and 2-TBA Reactants

Fluorescence spectra were measured with an Aminco-Bowman Spectrophotofluorometer (American Instruments Co., Inc.) combined with a Houston Instrument X-Y recorder. For the instrumental conditions used in this work 1 μg quinine sulfate per milliliter 0.1 N H_2SO_4 had fluorescence of 700.

Carbonyls formed during the peroxidation of arachidonic acid were measured as TBA reactants as described by Wills (7) with a few modifications. Samples of 1 ml were boiled for 15 min in the presence of 1 ml 0.67% TBA (Eastman Organic Chemicals) in 1 M acetic acid. The samples were cooled, and the absorbance was read at 532 nm.

Temperature-Denaturation Profiles

DNA samples were adjusted to a concentration of 0.4 A_{260} unit per milliliter in 0.1 x SSC solution, pH 6.8. Temperature-absorption characteristics were determined with a Gilford 2400 spectrophotometer equipped with a Haake thermoregulator-constant temperature circulator. The A_{260} values at each temperature were divided by the A_{260} at 30 C ($A_{260\,nm}{}^{t}/A_{260\,nm}$ 30 C).

Assay for Hydrolysis of Deoxyribonucleic Acid

DNA samples to be hydrolyzed by deoxyribonuclease I were dialyzed against a solution of 0.5 M Tris-HCl, pH 7.0, and 0.02 M $MgCl_2$. Samples that contained 80 μg DNA were hydrolyzed with 5 μg bovine pancreas DNase I (Worthington Biochemical Corp.) in 2.5 ml of the Tris-HCl buffer at 25 C. The increase in

absorbance at 260 nm was monitored with a Beckman DB spectrophotometer.

Template Activity of DNA Reacted with Peroxidizing Arachidonic Acid

Isolation of rat liver RNA polymerase followed the procedure described previously (6), which is a modification of the procedure described by Roeder and Rutter (8). The RNA polymerase assay mixture was the same as that described by Roeder and Rutter (9), except that ^{3}H-UTP (Amersham/Searle), specific activity 1 c/mmol, 0.13 M $(NH_4)_2SO_4$ and 0.2 mg enzyme protein were used. After incubation for 15 min at 37 C, the reaction was stopped by addition of 0.1 ml ice cold 0.1 M sodium pyrophosphate that contained 5 mM UTP and 2 mg/ml each of RNA and bovine serum albumin. The samples were quickly cooled and 0.1 ml of 5% sodium dodecyl sulfate was added, followed by addition of 2 ml of 10% trichloroacetic acid w/v that contained 40 mM sodium pyrophosphate. The acid insoluble material was collected on Whatman GF/C discs and washed eight times with 8 ml trichloroacetic acid-sodium pyrophosphate solution. The discs were dried, placed in 5 ml Aquasol (New England Nuclear) and the radioactivity determined in a liquid scintillation counter.

RESULTS

The fluorescence spectrum of the products formed in the 76 hr reaction of DNA with peroxidizing arachidonic acid, hereafter called DNA-arachidonate product, is shown in Figure 1. The excitation (315 nm) and emission (420 nm) maxima are somewhat different from those observed for DNA reacted with malonaldehyde, where excitation and emission maxima appeared at 390 nm and 460 nm, respectively (6). The fluorescence of the control DNA sample was very low and different from that of DNA-arachidonate product.

Figure 2 shows the formation of the fluorescent product and the amounts of TBA reactive materials present in the reaction mixture at various reaction times. After 12 hr of reaction a sharp increase in the fluorescent product was observed. At that time the TBA reactive materials reached a peak and gradually decreased as more fluorescent product formed. The decrease of TBA reactive materials could relate to its reaction with DNA, but only a small proportion would result in formation of fluorescent products. TBA reactive materials accumulated in the control sample of peroxidizing arachidonate, reached a peak at 37 hr of reaction, and then decreased after 46 hr.

A shift in the melting point and decrease in hyperchromicity of DNA as a result of reaction with peroxidizing arachidonate are shown in Figure 3. The melting point shifted from 70 C for the control sample to 65 C for the DNA-arachidonate product. The difference in susceptibility toward degradation by deoxyribonuclease between the DNA control and the DNA-arachidonate product is shown in Figure 4. Partial resistance against hydrolysis by deoxyribonuclease was observed for the DNA-arachidonate product.

Figure 5 shows the effect of the reaction of peroxidizing arachidonic acid with DNA on its template activity for RNA polymerase. A decrease in template activity at various DNA concentrations was observed. The substrate saturation points, ca. 5 μg DNA, were similar for both samples.

DISCUSSION

The formation of fluorescent products could result from the Schiff base complex formed by the reaction between the amino groups of the bases of DNA and the carbonyl end products of peroxidizing arachidonic acid. The fluorescence spectra of the DNA-arachidonate product differs from that of DNA reacted with malonaldehyde (6). Although malonaldehyde is one of the major products formed in oxidation of polyunsaturated fatty acids, other carbonyls may react with DNA, via conjugated Schiff base formation, to yield products with different fluorescence spectra.

Indications of the structural changes in DNA that resulted from reaction with peroxidizing arachidonic acid are seen from the decrease in the melting point, the decrease in hyperchromicity, partial resistance to hydrolysis by deoxyribonuclease and from the decrease in the template activity of the DNA-arachidonate product. Lipid peroxidation products seem to cause a partial disruption of the hydrogen bonding of DNA, probably by intrastrand crosslinking, thus lowering its melting point. Decreased hyperchromicity suggests that interstrand crosslinking also occurred and resulted in covalent linkages which are resistant to heat denaturation. Structural alterations of the double helix and partial denaturation caused a decrease in its susceptibility to hydrolysis by deoxyribonuclease and a decrease in the template activity for RNA polymerase. Similar results were observed when DNA was reacted with malonaldehyde (5,6). The substrate saturation points for RNA polymerase of both control DNA and DNA reacted with peroxidizing arachidonic acid were similar. This ob-

servation indicates that the RNA polymerase attachment sites of the reacted DNA remained intact. Studies with labeled arachidonic acid showed that lipid peroxidation breakdown products reacted mainly with the adenine and guanine and, to a lesser degree, with the cytosine bases of DNA. Whether lipid peroxidation plays a significant role in damage to DNA in vivo is still unknown and requires further studies.

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REFERENCES

1. Dahle, L.K., E.G. Hill and R.T. Holman, Arch. Biochem. Biophys. 99:253 (1962).
2. Kwon, T.W., and H.S. Olcott, J. Food Sci. 31:552 (1966).
3. Chio, K.S., and A.L. Tappel, Biochemistry 8:2821 (1969).
4. Chio, K.S., and A.L. Tappel, Ibid. 8:2827 (1969).
5. Brooks, B.R., and O.L. Klamerth, Eur. J. Biochem. 5:178 (1968).
6. Reiss, U., A.L. Tappel and K.S. Chio, Biochem. Biophys. Res. Commun. 48:921 (1972).
7. Wills, E.D., Biochem. J. 99:667 (1966).
8. Roeder, R.G., and W.J. Rutter, Proc. Nat. Acad. Sci., U.S., 65:675 (1970).
9. Roeder, R.G., and W.J. Rutter, Nature 224:234 (1969).

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Fluorescent Products of Phospholipids during Lipid Peroxidation

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ABSTRACT

During peroxidation, phosphatidyl ethanolamine and phosphatidyl serine formed fluorescent chromophores with maximum emission at 435 nm and maximum excitation at 365 nm. The development of fluorescence was related to formation of thiobarbituric acid reactive substances during lipid peroxidation. This relationship was studied by reacting dipalmityl phosphatidyl ethanolamine with the oxidation products of the methyl esters of arachidonic, linolenic and linoleic fatty acids. Reaction parameters affecting the development of lipid-extractable fluorescent chromophores are: the production of peroxidation products, especially malonaldehyde, from autoxidation of polyunsaturated fatty acids; the length of time these products react; and the availability of reactive amino groups on the phospholipids.

INTRODUCTION

Lipid peroxidation is a mechanism of cellular damage (1,2), and the major damage sites are subcellular organelle membranes. Mitochondrial and microsomal membranes contain relatively large amounts of polyunsaturated fatty acids in their phospholipids (3). These polyunsaturated fatty acids can undergo lipid peroxidation with the formation of free radical intermediates and carbonyl products (4). Malonaldehyde, one of the major carbonyls formed (5,6), was shown to react with amino compounds by Crawford et al. (7,8). More recently, it has been shown that malonaldehyde reacts with amines to form fluorescent chromophores (9,10). The relationship of formation of fluorescent chromophores to peroxidative damage was examined in the extracted lipid phase from organelle membranes that had been peroxidized in vitro (11). The intensity of the fluorescence was directly related to the degree of peroxidative damage.

This paper reports an investigation of amino phospholipid interaction with lipid peroxidation products in the formation of lipid soluble fluorescent chromophores and compares these products with those formed in peroxidized membranes.

EXPERIMENTAL PROCEDURES

Materials

Phosphatidyl ethanolamine and phosphatidyl choline were purchased from Supelco, and phosphatidyl serine from Schwarz/Mann; all were of animal origin. Synthetic dipalmityl phosphatidyl ethanolamine was obtained from Schwarz/Mann. The purity of all phospholipids was confirmed by thin layer chromatography. The methyl esters of arachidonic, linolenic and linoleic fatty acids were purchased from the Hormel Institute (99% purity). Malonaldehyde was obtained from J.T. Baker Chemical Co. as the 1,1,3,3-tetra ethoxypropane bis (diethyl acetal). 2-Thiobarbituric acid (TBA) was obtained from Sigma.

Natural Phospholipid Reaction Systems

Phosphatidyl ethanolamine, phosphatidyl choline and phosphatidyl serine were examined for the development of fluorescence during peroxidation. The reaction mixtures contained 2.0 mg phospholipid per milliliter of 10 mM sodium phosphate buffer, pH 7.4. The phospholipids were emulsified at room temperature for 10 min with a Sonic-Dismembrator (Quigley-Rochester, Inc.). Separate reaction flasks containing 12.5 ml of sample were used for each time interval at which TBA reactive

TABLE I

Production of Fluorescence and TBRS during Peroxidation of Amino Phospholipids

Phospholipid	Fluorescence[a]	TBA reactive substances[b]
Phosphatidyl ethanolamine	5.10	17.10
Phosphatidyl serine	0.45	5.85
Phosphatidyl choline	0.02	0.00

[a]Values calculated as fluorescence times meter multiplier setting per milligram phospholipid per 24 hr. The 0 hr fluorescence values have been subtracted from the 24 hr values.

[b]Measured as nmol MA/mg phospholipid at 24 hr.

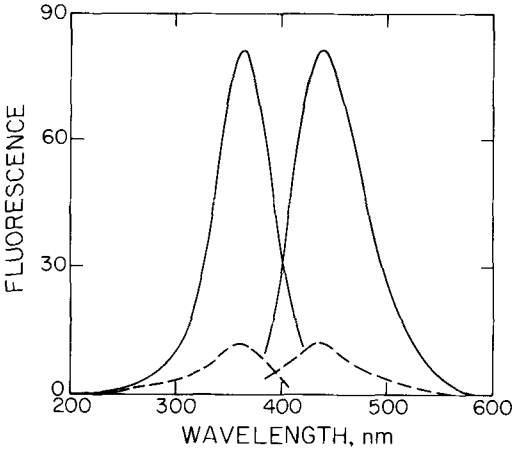

FIG. 1. Fluorescence spectra of phosphatidyl ethanolamine (——) and phosphatidyl serine (--) after 24 hr of peroxidation. The phospholipid concentration in the reaction was 2 mg/ml.

substances (TBRS) and fluorescence were measured. Each 25 ml flask was shaken in oxygen at 200 rpm in a 37 C water bath for varying time intervals.

Dipalmityl Phosphatidyl Ethanolamine Reaction Systems

Synthetic dipalmityl phosphatidyl ethanolamine (PEA) was gently suspended with a glass and Teflon homogenizer. The reaction systems contained 136 μmol PEA suspension and 310 μmol fatty acid methyl ester (arachidonate, linolenate or linoleate) in 18.0 ml 10 mM sodium phosphate buffer, pH 7.4. The samples in 50 ml flasks were shaken in oxygen at 200 rpm in a 37 C water bath. Samples were removed at varying time intervals up to 46 hr.

In a separate experiment, PEA was reacted with malonaldehyde (MA) prepared by the method of Kwon and Watts (12). MA, 2.0 μmol, was homogenized with 136 μmol PEA in 18 ml 10 mM sodium phosphate buffer, pH 7.4. The samples were shaken in 50 ml flasks at 200 rpm and 37 C for varying time intervals up to 46 hr. For all experiments, separate controls of PEA, fatty acid esters and MA were used.

PEA (50 μmol/ml) and methyl arachidonate (50 μmol/ml) were also reacted to compare the formation of fluorescence with development of TBRS. The samples were homogenized into a slurry with 1.5 ml 10 mM phosphate buffer, pH 7.4, and shaken in oxygen at 200 rpm and 37 C. Peroxidation was initiated by the addition of 0.25 ml each of 80 μM ascorbate and 80 μM $FeCl_3$.

Analytical Methods

Peroxidation was followed by the measurement of TBRS. An aliquot of 0.5 ml was mixed with 0.5 ml distilled water and 0.5 ml 30% trichloroacetic acid and centrifuged for 3 min. The supernatant portion was added to an equal volume of aqueous 0.67% TBA w/v and then heated in boiling water for 15 min. After cooling to room temperature, the absorbance of the samples was measured with a Beckman DB spectrophotometer at 532 nm.

Fluorescence was measured by extracting 2.0 ml aliquots of sample at room temperature with 4.0 ml chloroform-methanol 2:1 v/v for 1 min with a glass and Teflon homogenizer. Then 2 ml distilled water were added, the slurry mixed for 15 sec with a vortex mixer, and separated into two phases by centrifiguation. The chloroform layer was removed and examined for fluorescence. Fluorescence spectra were determined with an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Inc.) standardized with quinine sulfate (1 μg/ml in 0.1 N H_2SO_4), and were recorded on an X-Y recorder (Houston Instrument). The slit arrangement was 3, 1 and 3 mm for slits 3, 4 and 6, respectively. The sensitivity was set at 50. The meter multiplier setting was dependent upon the fluorescence intensity of each sample. With these instrument parameters,

TABLE II

Fluorescence and TBRS Produced by Dipalmityl Phosphatidyl Ethanolamine and Peroxidizing Unsaturated Fatty Acid Esters[a]

MA source	Relative fluorescence change[b]	TBA reactive substances[c]
Methyl arachidonate	0.512	177.0
Methyl linolenate	0.485	174.0
Methyl linoleate	0.027	1.7
Malonaldehyde	0.818	105.0

[a]Reaction system contained 7.5 μmol PEA and 17.2 μmol fatty acid or 0.1 μmol MA/ml.

[b]Values are calculated as fluorescence times meter multiplier setting per μmol PEA per 46 hr.

[c]nmol MA/ml in 46 hr.

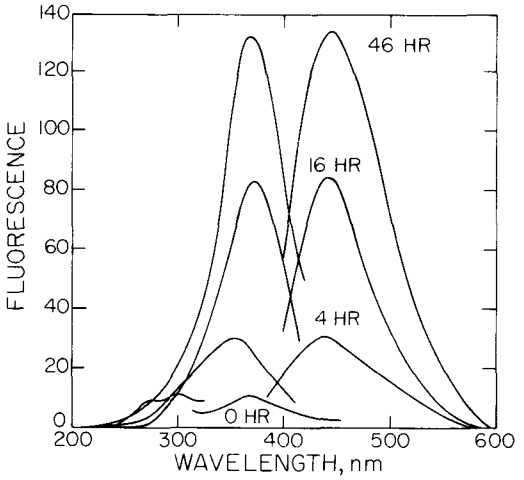

FIG. 2. Fluorescence spectra of dipalmityl phosphatidyl ethanolamine at 0, 4, 16 and 46 hr of reaction with peroxidizing methyl arachidonate. The TBRS were 0.002, 0.44, 0.54 and 0.62 mmol MA per mole arachidonate, respectively. The reaction contained 136 μmol PEA and 310 μmol methyl arachidonate in 18.0 ml 10 mM sodium phosphate buffer, pH 7.4.

the quinine sulfate standard had a fluorescence intensity of 71 at a meter multiplier setting of 0.3.

RESULTS

Data showing the relationship between formation in phospholipids of fluorescent products and extent of peroxidation, as measured by TBRS, are given in Table I. Phosphatidyl ethanolamine and phosphatidyl serine autoxidized and formed fluorescent products. Phosphatidyl choline neither autoxidized nor formed fluorescent chromophores.

In Figure 1 the fluorescence spectra of phosphatidyl ethanolamine and phosphatidyl serine are compared on samples peroxidized under identical conditions for 24 hr. The fluorescent chromophores had maximum emission at 435 nm and maximum excitation at 360 nm. As seen in this figure, phosphatidyl ethanolamine developed a greater amount of fluorescent product than phosphatidyl serine.

Dipalmityl phosphatidyl ethanolamine was reacted with different peroxidizing unsaturated fatty acid esters to determine the character of the fluorescent chromophores formed. Significant fluorescence was formed only in the presence of TBRS or MA (Table II). The lower production of TBRS during peroxidation of methyl linoleate correlates with low fluorescence. In the presence of purified MA, the greatest amount of fluorescent product was produced.

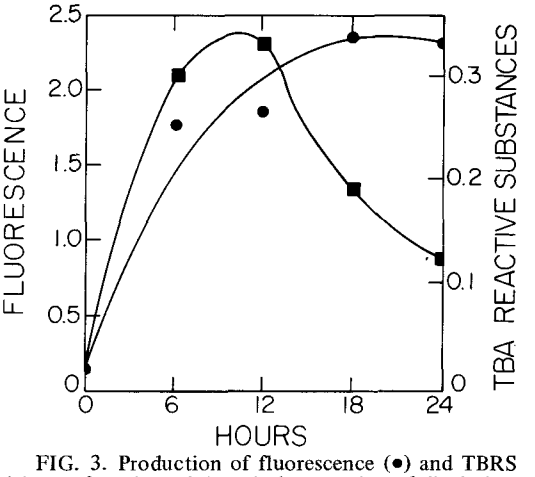

FIG. 3. Production of fluorescence (•) and TBRS (▪) as a function of time during reaction of dipalmityl phosphatidyl ethanolamine with oxidizing methyl arachidonate. TBRS are expressed as mmol MA/mol methyl arachidonate. Fluorescence is expressed as fluorescence times meter multiplier setting per μmol PEA.

The development of fluorescent PEA-arachidonate products and the spectral changes that occurred are shown in Figure 2. The PEA-arachidonate products had maximum fluorescence emission at 445 nm when excited at a maximum of 370 nm. Similar maximum fluorescence excitation and emission wavelengths were noted for the PEA-linolenate product. The PEA-MA product had a greater fluorescence intensity and exhibited higher maximum emission and excitation wavelengths, 475 nm and 400 nm, respectively.

To examine the rate of fluorescence development and production of TBRS, dipalmityl phosphatidyl ethanolamine was reacted with arachidonate at lower concentrations to optimize the oxidative process (Fig. 3). Amounts of TBRS from methyl arachidonate reached a maximum at 10 hr, while fluorescence development continued at a much reduced rate and reached a plateau after 18 hr.

DISCUSSION

The physical form of a phospholipid in an aqueous medium is greatly dependent on the degree of unsaturation and the charge on the phospholipid, and on the temperature, pH and ionic strength of the medium (13,14). Upon addition of water, phosphatidyl ethanolamine molecules increase their spacing to a greater extent than do corresponding phosphatidyl choline molecules (13). In addition, phosphatidyl choline forms tightly packed micelles with many concentric layers, while phosphatidyl ethanolamine forms much larger micelles that

contain greater volumes of water and that appear as sheets (14).

These observed differences in phospholipid micelle structures offer an explanation for the limited peroxidation of phosphatidyl choline. The more tightly packed structure is less accessible to oxygen, which hinders initiation of peroxidation and decreases the rate of propagation. However, even if peroxidation products had formed, fluorescence would not have developed because phosphatidyl choline lacks a reactive amino group.

The lipid soluble fluorescent chromophores that developed during peroxidation of phosphatidyl ethanolamine and phosphatidyl serine had fluorescence emission maxima in the region of 435-440 nm and excitation maxima in the region of 365-370 nm. Similar chromophores formed during peroxidation of unsaturated fatty acid esters in the presence of dipalmityl phosphatidyl ethanolamine. These chromophores are similar to those of rat liver mitochondrial and microsomal fractions peroxidized in vitro (11) and having an excitation maximum at 360 nm and an emission maximum at 430 nm. Earlier studies (9) showed that fluorescent chromophores formed by reaction of malonaldehyde with amino acids were N,N′-disubstituted 1-amino-3-iminopropenes, R-NH-CH=CH-CH=N-R′, with fluorescence emission maxima at 450-470 nm and excitation maxima at 350-400 nm. Peroxidation of subcellular organelle fractions produced aqueous fluorescent chromophores with similar spectral characteristics (15).

Among the products formed during lipid peroxidation are many aldehydes, ketones and alcohols (16). Of the carbonyls formed, MA has probably been studied most extensively (12,17) and has been widely used as an indicator of peroxidation of fatty acids (5,6), and of changes in foods (18) and membranes (1,11). The initial development of MA closely parallels other parameters of peroxidation, i.e., diene conjugation and oxygen uptake (19). MA is very reactive with amino acids (7-9), proteins (10,20) and aromatic amines (21).

In studies with PEA, the extent of fluorescence development was related to the amount of TBRS formed during peroxidation of fatty acid esters. MA was identified as a major component of the TBRS by its absorbance maximum at 532 nm (12). Corliss and Dugan (19) have shown a decrease in the amine content of phosphatidyl ethanolamine during lipid peroxidation, indicating reaction of the TBRS with the amino group.

The fluorescent chromophores formed during reaction of PEA with MA differed somewhat from those formed during reaction with TBRS of oxidizing fatty acid esters, in that they had higher wavelength maxima for emission and excitation, 475 and 400 nm, respectively. The fluorescence arises from families of chromophores (22). The spectral properties of the PEA-MA system could be related to fewer products being formed as compared to the systems containing many peroxidation products. The fluorescence intensity of the PEA-MA chromophores was the greatest, indicating that the presence of MA from the onset allowed a greater amount of product to be formed. In both peroxidizing microsomal membrane (23) and in the PEA-arachidonate systems, fluorescence developed only as peroxidation products increased. When the TBRS declined, the rate of fluorescent product formation decreased. Decrease in TBRS is related to decreased production, as the polyunsaturated fatty acids become fully peroxidized, and to the continued reactions of TBRS, including reactions with amines. These experiments indicate that formation of fluorescent phospholipid chromophores is directly related to the amount of peroxidation products present, to the length of time these products are allowed to react and to the availability of reactive amino groups on the phospholipids. The fluorescent chromophores formed from the amino phospholipids are similar to those found in the lipid extracts of peroxidized membranes.

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REFERENCES

1. Packer, L., D.W. Deamer and R.L. Heath, Advan. Gerontol. Res. 2:77 (1967).
2. Tappel, A.L., "N.Y. Academy of Sciences Conference on Vitamin E, 1971," Ann. N.Y. Acad. Sci. 203:12 (1972).
3. Fleischer, S., and G. Rouser, JAOCS 43:588 (1965).
4. Lillard, D., and E.A. Day, Ibid. 41:549 (1965).
5. Dahle, L.K., E.G. Hill and R.T. Holman, Arch. Biochem. Biophys. 98:253 (1962).
6. Tarladgis, B.G., and B.M. Watts, JAOCS 37:403 (1960).
7. Crawford, D.L., T.C. Yu and R.O. Sinnhuber, Agr. Food Chem. 14:182 (1966).
8. Crawford, D.L., T.C. Yu and R.O. Sinnhuber, J. Food Sci. 32:332 (1967).
9. Chio, K.S., and A.L. Tappel, Biochemistry 8:2821 (1969).
10. Chio, K.S., and A.L. Tappel, Ibid. 8:2827 (1969).
11. Dillard, C.J., and A.L. Tappel, Lipids 6:715 (1971).
12. Kwon, T.W., and B.M. Watts, J. Food Sci. 28:627 (1963).
13. Derivician, D.G., Progr. Biophys. Mol. Biol.

14:263 (1964).
14. Bangham, A.D., Ibid., 18:29 (1968).
15. Chio, K.S., U. Reiss, B. Fletcher and A.L. Tappel, Science 166:1535 (1969).
16. Keeney, M., in "Lipids and Their Oxidation," Edited by H.W. Schultz, E.A. Day and R.O. Sinnhuber, Avi Publishing Co., Inc., Connecticut, 1962, p. 79.
17. Kwon, T.W., and H.S. Olcott, J. Food Sci. 31:552 (1966).
18. Kwon, T.W., and B.M. Watts, Ibid. 29:294 (1964).
19. Corliss, G.A., and L.R. Dugan, Jr., Lipids 5:846 (1970).
20. Menzel, D.B., Ibid. 2:83 (1967).
21. Sawicki, E., T.W. Stanley and H. Johnson, Anal. Chem. 35:199 (1963).
22. Tappel, A.L., in "Pathological Aspects of Cell Membranes," Vol. 1, Edited by B.F. Trump and A. Arstila, Academic Press, New York, In press, 1973.
23. Bidlack, W.R., Ph.D. thesis, University of California, Davis, 1972.

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Nature of Fecal Sterols and Intestinal Bacterial Flora [1,2]

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ABSTRACT

Sterol excretion in the spontaneously atherosclerosis-susceptible White Carneau (WC) pigeon, the Silver King (SK) pigeon and the Show Racer (SR) pigeon was studied by thin layer chromatography (TLC), argentation TLC and gas liquid chromatography. Unlike man and the chicken, these pigeons excreted no coprostanol or coprostanone derivatives of sterols. Moreover incubation of ^{14}C-labeled cholesterol with pigeon feces indicated that, also unlike man and the chicken, these pigeons are unable to convert it to coprostanol. Bacteriologic examination revealed the absence of gram-negative anaerobic flora and of members of the genus *Bifidobacterium* in both the WC and SR pigeons. On the other hand, one of the two SK pigeons examined showed evidence of the presence of both *Bacteroides fragilis* and *B. bifidum* in the upper intestinal tract. Although no qualitative experiments were performed, no unusual characteristics of the aerobic flora were noted in these pigeons. In addition, analysis of human stool specimens indicated a "normal" bowel flora. The flora of the intestinal tract of the chicken is similar to that of the human. Because of this similarity, it appears that differences in environment (living conditions, diets) between the human and the chicken are of little consequence. The results obtained in this study suggest the possibility that the anaerobic gram-negative flora and possibly the *Bifidobacterium* may be responsible, at least in part, for the chemical conversion of cholesterol to coprostanol.

[1]Presented in part at the Fourth International Symposium on Drugs Affecting Lipid Metabolism, Philadelphia, September 1971, and at the AOCS Meeting, Los Angeles, April 1972.

[2]The following nomenclature has been used for the steroids referred to in this paper: cholesterol, cholest-Δ^5-en-3β-ol; coprostanol, 5β-cholestan-3β-ol; campesterol, 24-methylcholest-Δ^5-en-3β-ol; stigmasterol, 24-ethylcholest-$\Delta^{5,22}$-dien-3β-ol; β-sitosterol, 24-ethylcholest-Δ^5-en-3β-ol; coprocampestanol, 24-methyl-5β-cholestan-3β-ol; coprostigmastenol, 24-ethyl-5β-cholest-Δ^{22}-en-3β-ol; coprostigmastanol, 24-ethyl-5β-cholestan-3β-ol; coprostanone, 5β-cholestan-3-one; campestanone, 24-methyl-5β-cholestan-3-one; stigmastenone, 24-ethyl-5β-cholest-Δ^{22}-en-3-one; and β-sitostanone, 24-ethyl-5β-cholestan-3-one.

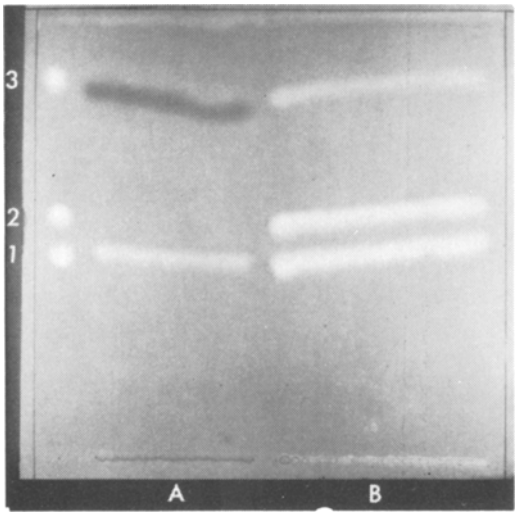

FIG. 1. Thin layer chromatographic separation of fecal sterols. (A) Pigeon feces. (B) Human feces. Standards: 1 = cholesterol; 2 = coprostanol; 3 = coprostanone. Adsorbent is Silica Gel G; solvent system, ethyl ether-heptane 55:45 v/v. Plates were stained with 2,7-dichlorofluoroscein spray in methanol.

INTRODUCTION

During studies (1) with the spontaneously atherosclerosis-susceptible White Carneau (WC) pigeon, it was noted that coprostanol and other 5β-stanols were absent from the feces of this species. In man, chicken and rodents, however, these stanols contribute nearly 50% of the total fecal sterols (2-4). These 5β-stanols have been shown to be formed by intestinal bacterial flora (5,6), although the specific bacteria which carry out this transformation have not been identified. The absence of 5β-stanols from the pigeon feces stimulated us to look for peculiarities in the intestinal bacterial flora of this species. For purposes of comparison, data were obtained from two additional pigeon species (Silver King and Show Racer), the chicken and man.

MATERIALS AND METHODS

All pigeons were obtained from the Palmetto Pigeon Plant (Sumter, S.C.). Their ages ranged from 8 months to 1 year, and they were housed two in a cage. Fecal samples were collected daily and kept frozen until analyzed for their biochemical properties.

Biochemical Methods

Cholesterol-4-^{14}C (specific activity, 60.9 mCi/mmol) was purchased from New England Nuclear (Boston, Mass.). Reference cholesterol, coprostanol, coprostanone, and plant sterols were obtained commercially from Schwarz/Mann Research Lab. (Orangeburg, N.Y.) and Applied Science Lab. (State College, Pa.). Their purity was assessed by thin layer (TLC) and gas liquid (GLC) chromatography.

The fecal steroids were extracted and purified as described previously (1), based on the procedures described by Miettinen et al. (7). Usually 2 g feces was refluxed with 20 ml 1N NaOH in 90% ethanol for 1 hr; 10 ml water was then added to the saponification mixture, and the neutral steroids were extracted thrice with 50 ml portions of petroleum ether. The petroleum ether fraction was evaporated to a small volume.

Steroid extracts of feces were subjected to TLC on Silica Gel G with the solvent system, ethyl ether-heptane, 55:45 v/v (7). This solvent system separated the 3-keto steroids and the 5β-stanols from the Δ^5-unsaturated sterols; however their 5α-stanols overlap with the Δ^5-unsaturated sterols. Reference cholesterol, coprostanol and coprostanone were used as markers. The bands were detected by spraying with 2,7-dichlorofluorescein in methanol. The bands corresponding to the markers were then scraped off into vials, and the steroids were eluted from the gel with four 4 ml portions of ethyl ether.

The 5α-stanols were separated from the corresponding mono-, di- and triunsaturated sterols on $AgNO_3$-impregnated Silica Gel G with the solvent system, chloroform-methanol-acetic acid 100:1:0.2 v/v/v (1,8). The bands were eluted from the gel with chloroform. During the entire procedure the losses were corrected for by using cholesterol-4-^{14}C as an internal standard. The average recovery of sterols from avian and human fecal samples was 95%.

After initial separation by TLC, the steroids were identified by GLC as their trimethyl silyl ethers. Their retention times were compared to those of cholesterol and plant sterol derivatives. An F&M 402 high efficiency gas chromatograph equipped with flame ionization detector was used. The sterols were chromatographed on glass columns packed with 3.8% W-98 on Diatoport (80-120 mesh). The operating conditions were: column, 230 C; injector, 260 C; detector, 250 C; carrier gas, helium (50 ml/min). The sterols were converted into their silyl ethers by the procedure described by Miettinen et al. (7) and quantitated by GLC

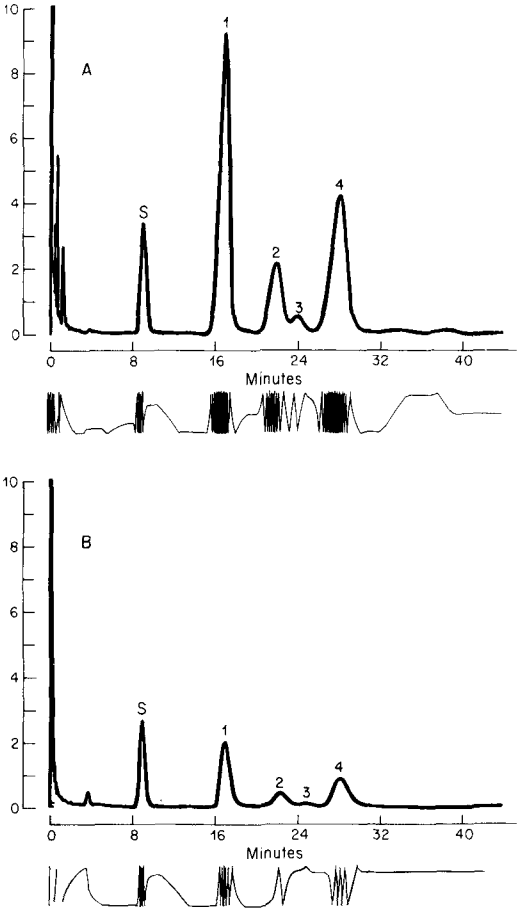

FIG. 2. Gas liquid chromatography of human fecal steroids as trimethyl silyl ethers. (A) 5β-Stanols. Peak identification: 1 = coprostanol; 2 = coprocampestanol; 3 = coprostigmastenol; 4 = coprostigmastanol. (B) 5β-Stan-3-ones. Peak identification: 1 = coprostanone; 2 = campestanone; 3 = stigmastenone; 4 = β-sitostanone. In both, peak S represents internal standard, 5α-cholestane; column conditions as described in text.

with 5α-cholestane as an internal standard. Quantitative response was obtained for all steroids on GLC under the conditions used.

Identifications of the sterols were based on their migration (R_f values) during TLC on Silica Gel G or $AgNO_3$-impregnated Silica Gel G and during GLC (1,4,8).

Fecal specimens were diluted with physiologic saline and homogenized; cholesterol-4-^{14}C in 25 μl ethanol was added to the homogenate and the mixture was incubated anaerobically at 37 C for 7 days (5,9). Control tubes contained either an excess of ethanol or no stool. The steroids were extracted as described above. If radioactivity was found in the region corresponding to coprostanol, this was subjected to rechromatography and crystallized to constant

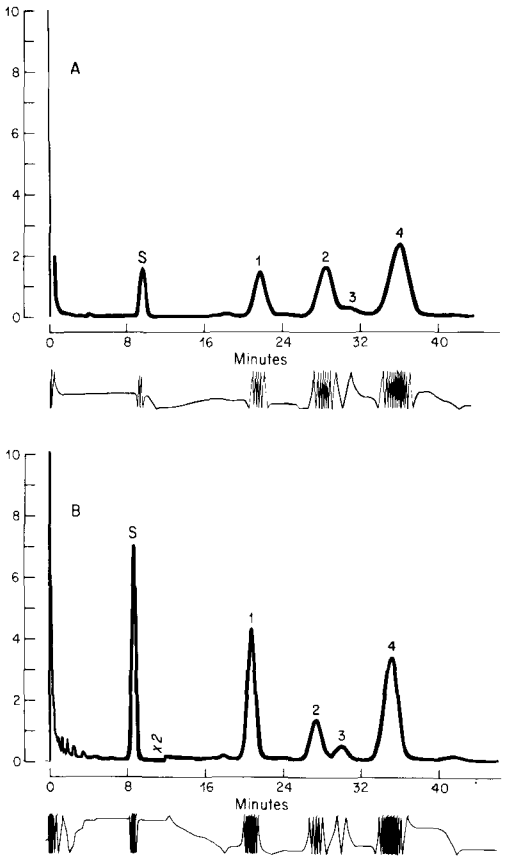

FIG. 3. Gas liquid chromatography of human (A) and pigeon (B) Δ^5-sterols. Peak identification: 1 = cholesterol; 2 = campesterol; 3 = stigmasterol; and 4 = β-sitosterol. Peak S represents internal standard, 5α-cholestane. Column conditions as described in text.

specific activity with authentic compounds to prove its identity.

Bacteriologic Methods

The pigeons and chickens were killed with an overdose of barbital. The abdominal cavity was opened under aseptic conditions and the entire intestinal tract was removed *en bloc*. The tract was cut into segments to correspond to the human duodenum, upper and lower small intestine, large intestine and distal cloaca. The ceca of the chicken was also included for analysis. A portion of the intestinal contents from each section (starting with the duodenum and proceeding distally) was removed aseptically and placed in a sterile screw-cap vial which had been flushed with CO_2. These specimens were received in the laboratory for processing within 30 min.

Specimens of human stool were collected in new 1 gal paint cans that had been previously flushed with CO_2. None of these specimens were obtained from individuals receiving antibiotics or chemotherapy. All stool specimens were submitted to the laboratory for subsequent processing as quickly as possible (within 1.5 hr).

All specimens were examined for both aerobic and anaerobic organisms. Anaerobic bacteria were isolated by the modified Gas-Pak (BBL) system incorporating the holding-jar procedure as described by Martin (10); this method precludes isolation of the highly oxygen-intolerant anaerobes. Sheep blood agar (5%), phenylethyl alcohol-blood agar, and kanamycin-vancomycin-blood agar supplemented with vitamin K and hemin were used as plating media. In addition, thioglycollate 135-C (BBL) was used as the enrichment broth. All anaerobic plating media were stored until use—no longer than 72 hr—under a constant flow of CO_2 in a relatively airtight cabinet (10). After 48-72 hr of incubation, plates were inspected, and representative colonies were selected for subculture and subsequent identification. An additional blood agar plate was incubated under 5-10% CO_2 to provide an index of the total bacterial flora present. The selection of colonies was not done under anaerobic conditions.

All negative broths were examined periodically for growth, and none were discarded until 14 days after inoculation. The designation of "no growth" was determined by inspection, Gram stain and subculture. Identification was based on Gram stain characteristics, colonial morphology, biochemical reactions and gas chromatography according to previously described methods (11-14).

All aerobic cultures were inspected after 18-24 hours of incubation at 35 C. Sheep blood agar (5%), eosin-methylene blue agar and phenylethyl alcohol-blood agar were used as plating media. Definitive identification of these bacteria was based on standard methods (15).

Bird seed medium (16) also was included in these studies in order to detect strains of *Cryptococcus neoformans* (as well as other yeast forms).

RESULTS

Biochemistry

Virtually no coprostanol or coprostanone derivatives were found in pigeon feces (Fig. 1); both these derivatives are found to a significant extent in human feces. The dark band below coprostanone in pigeon feces was identified by GLC as α-tocopherol. The fractions corresponding to authentic coprostanol and coprostanone were eluted from the gel and subjected to GLC

as their trimethyl silyl ethers (Fig. 2). The 5β-stanol fraction was made up of both coprostanol and other 5β-stanols of the plant sterols, which were identified as coprocampestanol, coprostigmasterol and coprostigmastanol by comparing their retention times with those of authentic standards. The 3-keto steroid fraction corresponding to coprostanone gave peaks identified as coprostanone, campestanone, stigmastenone and β-sitostanone. The nature of the sterols present in 5β-stanol and 3-keto steroid fractions from the chicken was similar to that of man (4). In the humans the 5β-stanols and 3-keto steroids contributed 41.5 and 12.5% of the total sterols while in the chicken they contributed 3.5 and 1.5%; the rest was made up of Δ^5-sterols. The Δ^5-sterol patterns of both pigeon and man were fairly similar (Fig. 3). The compounds identified in this fraction were cholesterol, campesterol, stigmasterol and β-sitosterol. These sterols were also the major sterols found in chicken feces (4). A detailed quantitative study of the pigeon and chicken fecal sterols has been published elsewhere (1,4). Cholestanol and other 5α-stanols were found in feces of both species, although the White Carneau pigeon excreted up to 36% of the total sterols as 5α-stanols.

Further experiments were done to compare the ability of feces from pigeons and humans to form coprostanol (the conversion of cholesterol into coprostanol has been successfully demonstrated in humans) (5,6). Human feces converted cholesterol into coprostanol to an extent of 66% (over the blank), while in the pigeon no conversion was noted (Table I). The conversion of cholesterol into coprostanol was noted in all specimens of human fecal samples examined. Incubation of cholesterol-^{14}-C with chicken feces showed that it can also convert cholesterol into coprostanol.

TABLE I

Incubation of Cholesterol-4-^{14}C with Pigeon and Human Feces[a]

Incubation	Experiments, no.	% Conversion to coprostanol,[b] mean ± SD
Pigeon		
Blank	10	0.39 ± 0.20
Experimental	6	0.30 ± 0.16
Human		
Blank	6	1.39 ± 0.98
Experimental	6	68.70 ± 6.70

[a]Substrate cholesterol-4-^{14}C: 2 to 4 x 10^5 dpm with pigeon feces and 5 x 10^4 dpm with human feces.

[b]Conversion was calculated after separation of coprostanol from cholesterol by thin layer chromatography and determination of its radioactivity.

Bacteriology

The ten pigeons studied included six WC pigeons (three males and three females), two SR pigeons (both males), and two SK pigeons (both females). One WC and two SR pigeons had been fasting for 24 hr. The ages ranged from 8 months to 1 year for six of the birds; ages were not available for the remaining four.

Tables II-IV present data on the aerobic and anaerobic bacteria isolated from the various levels of the intestinal tract of the pigeons. Although quantitative experiments were not performed during this study, the organisms isolated most frequently from all three species of pigeons were the anaerobic bacteria, *Eubacterium lentum* and *Clostridium perfringens*. Of interest is the additional recovery of *Eubacterium aerofaciens* from three of the WC pigeons (WC-3, WC-8 and WC-35). Generally speaking, there was relatively equal distribution of these anaerobic bacteria in all five levels of the intestinal tract.

The most significant finding was the complete lack of a gram-negative anaerobic flora in both the WC and SR pigeons. Moreover no members of the genus *Bifidobacterium* were isolated from these two species of birds. On the other hand, one of the two SK pigeons (Table III, SK-54) showed evidence for the presence of both *Bacteroides fragilis* and *B. bifidum* in the upper intestinal tract. The pH of the contents of the small and large intestine of the three species of pigeons ranged from 7.2 to 7.4. It would appear that suppression of the gram-negative anaerobic microflora might be related to factors other than pH, such as the gastric or jejunal mucosa (17).

Bacterial analysis of the intestinal contents from a fasting female chicken (1 year old) was also included in this study. Six levels of the chicken's intestinal tract, including the ceca, were studied (Table V). The flora of chicken and man were similar, whereas those of chicken and pigeon were markedly different even though the diets and living conditions of these two species were similar. This suggests that diet and living conditions are not significant factors affecting intestinal flora.

For comparison, ten adult stool samples obtained from hospitalized patients not receiving antimicrobial therapy were analyzed for their bacterial flora. Four of these patients were considered normal—not receiving drugs and without prior treatment for atherosclerosis. The six other patients were undergoing follow-up of treatment for atherosclerosis. Of the ten stool specimens submitted, eight also were

TABLE II

Organisms Isolated from Gastrointestinal Tract of Six White Carneau Pigeons

Site	Anaerobes		Aerobes	
	Organism	No.[a]	Organism	No.[a]
Duodenum	*Clostridium perfringens*	3	*Bacillus* sp.	6
	Clostridium tetani	1	*Corynebacterium* sp.	1
	Eubacterium aerofaciens	1	*Escherichia coli*	3
	Eubacterium lentum	3	Lactobacilli	4
	Eubacterium quartum	1	*Staphylococcus epidermidis*	3
	Lactobacilli	1	Streptococcus, grp. D	1
			Streptococcus, viridans grp.	1
			Yeast	3
			Staphylococcus aureus	1
Upper small intestine[b]	*Clostridium perfringens*	2	*Bacillus* sp.	6
	Clostridium tetani	1	*Escherichia coli*	5
	Eubacterium aerofaciens	1	Lactobacilli	5
	Eubacterium lentum	3	*Staphylococcus aureus*	1
			Staphylococcus epidermidis	2
			Yeast	3
Lower small intestine[b]	*Clostridium perfringens*	1	*Bacillus* sp.	6
	Eubacterium aerofaciens	1	*Escherichia coli*	4
	Eubacterium lentum	4	Lactobacilli	5
	Peptococcus prevotii	1	*Staphylococcus epidermidis*	3
			Streptococcus, viridans grp.	1
			Unidentified gram-neg. bacillus (enteric)	1
			Yeast	3
Large intestine[c]	*Actinomyces naeslundii*	1	*Bacillus* sp.	6
	Clostridium perfringens	2	*Escherichia coli*	5
	Clostridium tetani	1	Lactobacilli	5
	Eubacterium aerofaciens	1	*Staphylococcus epidermidis*	1
	Eubacterium lentum	4	Streptococcus, viridans grp.	2
			Yeast	4
Distal cloaca	*Clostridium perfringens*	2	*Bacillus* sp.	4
	Eubacterium aerofaciens	2	*Escherichia coli*	5
	Eubacterium lentum	6	Lactobacilli	4
			Staphylococcus aureus	1
			Staphylococcus epidermidis	1
			Streptococcus, viridans grp.	4
			Yeast	5

[a]Number of pigeons from which organism was isolated.

[b]No anaerobes isolated from two pigeons.

[c]No anaerobes isolated from one pigeon.

processed for their ability to convert cholesterol into coprostanol. *B. fragilis* was present in all specimens, and *Cl. perfringens* was present in seven. *Peptococcus* and *E. aerofaciens* were isolated from five specimens. Other anaerobic organisms isolated less frequently included several species of *Bifidobacterium*, *Eubacterium*, *Clostridium*, *Lactobacillus*, *Peptostreptococcus*, *Fusobacterium*, *Bacteroides*, *Propionibacterium* and *Veillonella*. Quantitative determinations of the relative number of bacteria present in each specimen were not performed. However no discernible differences or trends were noted regarding the anaerobic bacteria isolated.

DISCUSSION

It has been established that the predominant bacteria in feces are anaerobes (18-20). Several recent studies have shown that the bacterial flora of the normal intestinal tract of man is for the most part consistent and exists in a fairly logical sequence. For example, the lactobacilli usually dominate in the stomach, whereas in the small intestine the predominant bacteria are enterococci and lactobacilli (21-23). Although the lactobacilli and enterococci are present in the large intestine in similar concentrations (10^6-10^8/g content), they no longer are the predominant flora. Indeed, they appear to make up ca. 0.1% of the flora of this part of the intestinal tract, where there are 10^{10}-10^{11} organisms per gram of content. In the large intestine, only 5-10% of the bacteria are members of the family Enterobacteriaceae or other facultative bacteria; the obligate anaerobes

TABLE III

Organisms Isolated from Gastrointestinal Tract of Two Show Racer Pigeons

Site	Anaerobes		Aerobes	
	Organism	No.	Organism	No.
Duodenum[a]	*Eubacterium lentum*	1	*Escherichia coli*	2
	Eubacterium quintum	1	Lactobacilli	1
			Staphylococcus epidermidis	1
			Streptococcus, viridans grp.	1
Upper small intestine	*Clostridium perfringens*	1	*Escherichia coli*	2
	Eubacterium lentum	1	Lactobacilli	1
Lower small intestine	*Clostridium perfringens*	1	*Escherichia coli*	2
	Eubacterium lentum	1	Streptococcus, viridans grp.	1
Large intestine	*Clostridium perfringens*	1	*Bacillus sp.*	1
	Eubacterium quintum	1	*Escherichia coli*	2
	Lactobacillus catenaforme	1	Lactobacilli	1
Distal cloaca	*Clostridium perfringens*	1	*Escherichia coli*	2
	Eubacterium lentum	1	Streptococcus, grp. D	1
	Eubacterium quintum	1	Streptococcus, viridans grp.	1

[a]No anaerobes isolated from one pigeon.

make up the vast majority of the organisms that are part of the normal flora (21).

Investigations on two healthy men as well as those on other humans, hogs, dogs, chickens and rats suggested that, in general, the same kinds of bacteria are present in feces of all of these subjects (21). However various kinds of bacteria may be present in different proportions. In that study, in any one of the healthy men the relative proportions appeared to remain remarkably constant over long periods. Furthermore greater differences were found in the intestinal flora of different animals of the same species, including littermate hogs, than in the flora of different species.

The anaerobic bacteria that predominate in the intestinal flora include many species of bifidobacteria, bacteroides, eubacteria, propionibacteria, peptostreptococci and others. It has been demonstrated that *B. fragilis* is the most common organism, with the *Bifidobacterium* being the second most prevalent in the normal fecal flora (22-24). Others have confirmed these observations, with the exception that the second most prevalent organisms appeared to be members of the genus *Eubacterium* (21).

The anaerobic bacteria isolated from the WC pigeons, the SR pigeons and one of the SK pigeons revealed an obvious lack in the gram-negative flora with the methods of isolation used in this study. Members of the genus *Bifidobacterium* also were not detected in these pigeons. Particularly with the WC and SR pigeons, the apparent lack of these bacteria seems to be a consistent observation, since sex, fasting, presence of round worms, region of GI tract sampled and age appeared to have no observable effect on the flora. The inability to isolate either of these major groups of anaerobes in nine of ten pigeons appears to be in direct contrast to the presence of these bacteria in humans, chickens and other animals. On the other hand, the anaerobic isolates obtained from feces of the human subjects in this study agree with those reported by others (18-24), although no quantitative confirmation was obtained. Moreover the aerobic flora from all of the pigeons and from man indicated no significant qualitative differences in any of the bacteria that were recovered.

Conversion of cholesterol to coprostanol by intestinal bacteria of animals has been demonstrated both in vivo and in vitro with fecal suspensions (25-27). Although coprostanol has been presumed to be formed mainly in the colon, there has been no definite evidence in this regard. In view of the fact that significant numbers of bacteria are present in the small intestine, it would be interesting to know the relative ability of these bacterial species from various sites of the intestinal tract to carry out this reaction. Some attempts have been made to locate the specific bacteria that carry out the formation of coprostanol. Anaerobes have been found to be most effective in this conversion (26); however it also was noted that certain strains of *Cl. perfringens, Cl. sporogenes, Bifidobacterium* and *E. coli* were inactive in this conversion. This would suggest that more than one species of bacteria (either directly or indirectly) may be involved or that optimal conditions for this conversion have not been met. However the demonstration of direct

TABLE IV

Organisms Isolated from Gastrointestinal Tract of Two Silver King Pigeons

Site	Anaerobes		Aerobes	
	Organism	No.	Organism	No.
Duodenum	*Bifidobacterium bifidum*	1	*Bacillus* sp.	2
	Clostridium perfringens	2	Lactobacilli	2
	Eubacterium lentum	1	*Staphylococcus epidermidis*	2
	Lactobacillus catenaforme	1	Yeast	1
			Streptococcus, grp. D	1
Upper small intestine	*Bacteroides fragilis*	1	*Bacillus* sp.	2
	Bifidobacterium bifidum	1	Lactobacilli	1
	Eubacterium lentum	2	Streptococcus, grp. D	2
	Eubacterium quartum	1	Yeast	1
Lower small intestine	*Bifidobacterium bifidum*	1	*Bacillus* sp.	2
	Eubacterium lentum	1	Lactobacilli	2
	Eubacterium quartum	1	Streptococcus, grp. D	1
			Yeast	1
Large intestine	*Clostridium* sp.	1	Bacilli, gram-neg., not viable	1
	Eubacterium lentum	1	*Bacillus* sp.	2
	Eubacterium quartum	1	Lactobacilli	2
			Yeast	1
			Streptococcus, grp. D	1
Distal cloaca	*Eubacterium aerofaciens*	1	Bacilli, gram-neg., not viable	1
	Eubacterium lentum	2	*Bacillus* sp.	2
	Eubacterium quartum	1	*Escherichia coli*	1
	Lactobacillus crispatus	1	Lactobacilli	2
			Streptococcus, grp. D	1
			Yeast	2

saturation of the $\Delta^{5,6}$ double bond as the major pathway of coprostanol formation indicates that a single enzyme must be associated with at least one species of bacteria. The unavailability of the specific strain of the bacteria responsible for this conversion has been the main reason that no attempts have been made to purify the enzyme catalyzing this reaction (Δ^5-sterol-5β reductase).

The results of our study suggest the possibility that the anaerobic gram-negative flora, and possibly the *Bifidobacterium*, may at least in part be responsible for the chemical conversion of cholesterol to coprostanol. Studies are in progress to determine exactly which anaerobic bacteria may be responsible for this conversion and the quantity of bacteria necessary to achieve the conversion. Experiments are planned to take advantage of a complete anaerobic environment for a more detailed study of the oxygen-sensitive flora that appear to be closely linked to the gastrointestinal mucosa.

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REFERENCES

1. Subbiah, M.T.R., B.A. Kottke and I.A. Carlo, Mayo Clin. Proc. 45:729 (1970).
2. Wells, W.W., and M. Makita, Anal. Biochem. 4:204 (1962).
3. Eneroth, P., K. Hellström and R. Ryhage, J. Lipid Res. 5:245 (1964).
4. Subbiah, M.T.R., B.A. Kottke and P.E. Zollman, Comp. Biochem. Physiol. 41:695 (1972).
5. Rosenfeld, R.S., D.K. Fukushima, H.L. Hellman and T.F. Gallagher, J. Biol. Chem. 211:301 (1954).
6. Rosenfeld, R.S., and T.F. Gallagher, Steroids 4:515 (1964).
7. Miettinen, T.A., E.H. Ahrens, Jr., and S.M. Grundy, J. Lipid Res. 6:411 (1965).
8. Subbiah, M.T.R., B.A. Kottke and I.A. Carlo, Lipids 6:517 (1971).
9. Wood, P.D.S., and D. Hatoff, Ibid. 5:702 (1970).
10. Martin, W.J., Appl. Microbiol. 22:1168 (1971).
11. Smith, L. DS., and L.V. Holdeman, "The Pathogenic Anaerobic Bacteria," Charles C. Thomas, Springfield, Ill., 1968, p. 423.
12. Willis, A.T., "Anaerobic Bacteriology in Clincial Medicine," Butterworth & Co., London, 1960, p. 163.
13. Dowell, V.R., Jr., and T.M. Hawkins, "Laboratory Methods in Anaerobic Bacteriology in NCDC Laboratory Manual," USDHEW, Public Health Service Publication 1803, Washington D.C., Government Printing Office, 1969, p. 33.
14. "Outline of Clinical Methods in Anaerobic Bacteriology," Second review, Virginia Polytechnic Institute and State University Anaerobe Lab., Blacksbury Va., 1970.
15. Blair, J.E., E.H. Lennette and J.P. Truant, "Man-

TABLE V

Organisms Isolated from Gastrointestinal Tract of One Chicken

Site	Anaerobes	Aerobes
Upper small intestine	*Eubacterium lentum*	Lactobacilli *Staphylococcus epidermidis*
Lower small intestine	*Eubacterium lentum* *Lactobacillus crispatus*	*Bacillus* sp. Lactobacilli *Staphylococcus epidermidis*
Colon	*Actinomyces viscosus* *Bacteroides fragilis* *Bacteroides oralis* *Eubacterium cylindroides* *Eubacterium lentum* *Peptococcus* sp. *Peptostreptococcus* sp. *Propionibacterium granulosum*	*Bacillus* sp. *Corynebacterium* sp. *Escherichia coli* Lactobacilli Streptococcus, grp. D
Distal cloaca	*Bacteroides oralis* *Clostridium perfringens* *Eubacterium lentum* *Veillonella* sp.	*Bacillus* sp. *Escherichia coli* *Staphylococcus epidermidis* Streptococcus, grp. D *Streptococcus*, viridans grp.
Cecum	*Bacteroides hypermegas* *Bacteroides oralis* *Bifidobacterium bifidum* *Eubacterium cylindroides* *Eubacterium lentum* *Peptococcus* sp.	*Bacillus* sp. *Escherichia coli* *Sarcina* Streptococcus, viridans grp.

ual of Clinical Microbiology," American Society for Microbiology, Bethesda, Md., 1970, p. 727.
16. Shields, A.B., and L. Ajello, Science 151:208 (1966).
17. Gorbach, S.L., Gastroenterology 60:1110 (1971).
18. Eggerth, A.H., and B.H. Gagnon, J. Bacteriol. 25:389 (1933).
19. Haenel, H., and W. Müller-Beuthow, Zentralbl. Bacteriol. (Naturwiss) 167:123 (1956).
20. Haenel, H., J. Appl. Bacteriol. 24:242 (1961).
21. Moore, W.E.C., E.P. Cato and L.V. Holdeman, J. Infect. Dis. 119:641 (1969).
22. Drasar, B.S., J. Pathol. Bacteriol. 94:417 (1967).
23. Finegold, S.M., and L.G. Miller, Amer. Soc. Microbiol. (Bacteriol. Proc.) 1968:93.
24. Finegold, S.M., D.J. Posnick, L.G. Miller and W.L. Hewitt, Ernahrungsforschung 10:316 (1965).
25. Dam, H., Biochem. J. 28:515 (1934).
26. Snog-Kjaer, A., I. Prange and H. Dam, J. Gen. Microbiol. 14:256 (1956).
27. Coleman, D.L., and C.A. Baumann, Arch. Biochem. Biophys. 72:219 (1957).

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Observations on Changes in Lipid Composition and Lecithin-Cholesterol-Acyl Transferase Reaction of Bovine Plasma Induced by Heat Exposure

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ABSTRACT

A study has been made with steers maintained at 22 C and 35 C on the lipid composition of the plasma, with particular reference to the role and specificity of the lecithin-cholesterol-acyl transferase enzyme in the formation of the cholesteryl esters. Exposure to a temperature of 35 C produced a steady decline in the total lipid concentration of the plasma, which reached a new equilibrium after 7-8 days. The concentrations of the lecithin, free cholesterol and cholesteryl ester fractions in the plasma of the steers at 35 C were reduced to ca. 60% of the corresponding concentrations found in the animals maintained at 22 C. The concentration of 18:2 circulating in both the cholesteryl ester and lecithin fractions was reduced particularly by exposure to the elevated temperature. No significant change in total blood volume could be detected in the animals between the two environmental temperatures. Incubation of the plasma at 38 C for 20 hr resulted in an increase in the concentrations of the fatty acids contained in the cholesteryl ester and lysolecithin fractions and a decrease in the concentration of the fatty acids contained in the lecithin fraction for the animals at both environmental temperatures. The net esterification of cholesterol by the acyl transferase enzyme in the plasma from the animals at 35 C was very much less than that found in the plasma from the animals at 22 C. Under the conditions of the experiment, the esterification process was shown to have a high specificity for 18:2. Evidence is available to suggest that the relationship between the acyl transferase activity of the plasma and the polyunsaturated fatty acids of the circulatory lipids may be severely disturbed by high environmental temperatures. These results are discussed in relation to several known metabolic effects observed in animals exposed to high environmental temperatures.

INTRODUCTION

Breed can have a significant effect upon the plasma lipid composition of cattle, and evidence now exists to suggest that the ability to adapt to physiological stress may be related to the lipid composition of the plasma (1). It is known that British breeds of cattle do not readily adapt to conditions of heat stress and that, compared with Zebu cattle, their performance under such conditions is considerably below their genetical potential (2). Analysis of the plasma lipid composition of various breeds of cattle (1) has shown that both the free and esterified cholesterol, phospholipid and total lipid concentrations are significantly lower in British breeds than in Zebu breeds of cattle. Although the work of Diven et al. (3) has shown that, under conditions of high environmental temperatures, the concentration of cholesterol in the plasma of the bovine was significantly reduced, very little information appears to be available on the direct effect of exposure to heat on the lipid constituents of the plasma. In cattle the cholesteryl ester and phospholipid fractions comprise by far the major portion of the lipids present in the plasma and together can constitute up to 83% of the total lipid present (4). As in many other animal species, it has been shown that in cattle the plasma lecithin-cholesterol-acyl transferase (LCAT), which catalyzes the reaction between the fatty acids in the β-position of lecithin and the free hydroxyl group cholesterol, is of central importance in the formation of the plasma cholesteryl esters (5).

In the present paper results are presented on the direct effect of exposure to a high environmental temperature on the plasma lipid composition of the bovine. Net esterification of cholesterol by the LCAT enzyme in the plasma under these conditions has been measured, and an attempt has been made to evaluate the role of the LCAT system in the changes in the plasma lipid composition that are shown to occur.

METHODS

Animals and Diet

The animals used were eight steers of the

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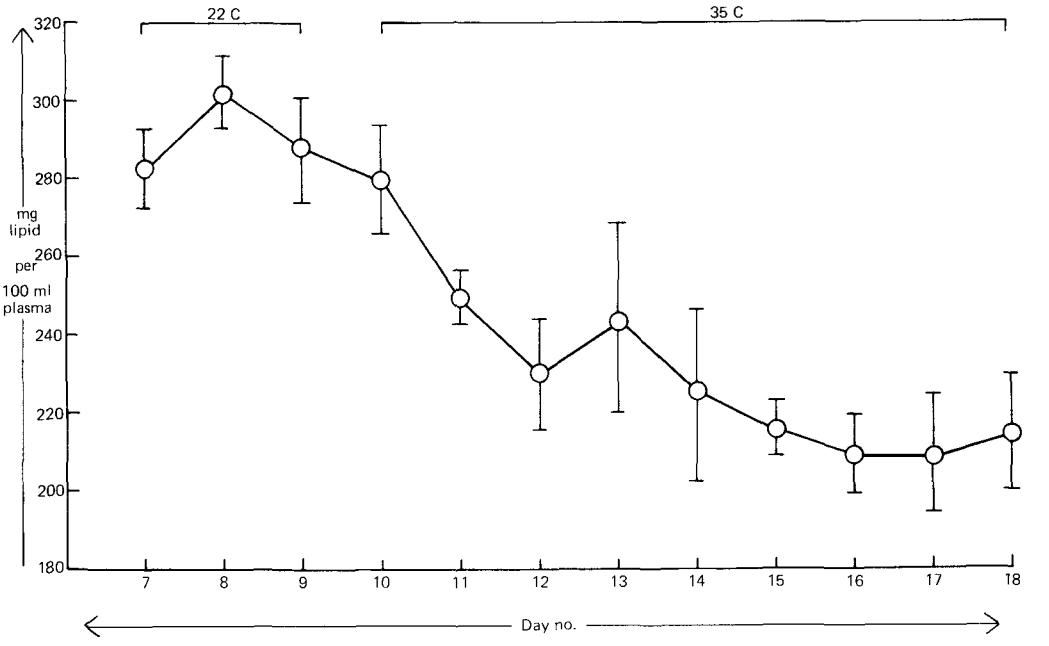

FIG. 1. The effect of the change in environmental temperature (22-35 C) upon the total plasma lipid concentration (mg/100 ml plasma) of steers.

Ayrshire breed. Their age was ca. 15 months and their weights ranged between 218 and 255 kg. The animals were housed in individual stalls in a controlled temperature room. Each animal received twice daily at 0600 hr and 1500 hr 3 kg of a diet consisting of 2 parts w/w of hay and 1 part w/w of a proprietary concentrate mixture. Water was available ad libitum.

Procedure

After a preliminary period of 2 weeks on the diet, the animals were maintained for 9 days at an initial temperature of 22 C. The temperature of the room was then increased to 35 C and maintained at this higher temperature for a further 9 days, i.e., days 10-18). Rectal temperatures were recorded daily for each animal.

In experiment 1 a polythene cannula, external diameter 2 mm, was aseptically inserted under local anaesthesia into the external jugular vein of four animals. Using a 3.8% w/v solution of sodium citrate as an anticoagulant, ca. 60 ml blood was obtained from each of the animals at 1000 hr on each of days 7-18. Approximately 15 min prior to the blood sampling, 6 ml of a 0.05% w/v solution of Evans Blue dye in saline was introduced into the circulation through the cannula. After centrifugation of the blood, ca. 20 ml plasma was retained for lipid analysis according to the methods outlined below. The remainder of the plasma was then used for the extraction of the dye by the method of Campbell et al. (6) and the total blood volume determined according to the dye dilution technique of Nitshe and Cohen (7).

In experiment 2 ca. 120 ml of blood was obtained at 1000 hr from the jugular vein of the remaining four animals on days 8, 9, 17 and 18 by means of Vacutainer tubes (Becton Dickinson, Rutherford, N.J.) that had been chilled to 4 C and contained heparin as an anticoagulent. Before use, all glassware was sterilized in a hot air oven. After centrifugation of the blood at 4 C, 10 ml aliquots of the plasma were transferred to 50 ml Ehrlenmeyer flasks and incubated at 38 C on a shaking water bath. Duplicate plasma samples from each of the animals were incubated for 20 hr. Appropriate volumes of unincubated plasma were added directly to methanol to provide control samples. Further control samples were obtained by incubating plasma in which the LCAT enzyme had been inactivated by heating for 30 min at 65 C (8). Analysis revealed no significant difference between the separate control samples.

Extraction of Lipids and Methods of Analysis

The total lipids were extracted from the plasma samples by the method of Nelson and Freeman (9). In experiment 2 the lipid extracts were then chromatographed on columns of

TABLE I

Effect of Incubation for 20 hr at 38 C on Concentrations[a] of Lecithin, Lysolecithin, Free Cholesterol and Cholesterol Ester Fractions in Bovine Plasma from Animals Maintained at 22 C and 35 C

	mg Fatty acid per 100 ml plasma		mg Lipid per 100 ml plasma		μMol per ml plasma		Per cent change	
Lipid	22 C[b]	35 C[b]	22 C[b]	35 C[b]	22 C[b]	35 C[b]	22 C[b]	35 C[b]
Lecithin								
Control plasma	73.3[b]	50.9	100.5[b]	69.8	1.32[b]	0.92		
Incubated plasma	56.2[b,c]	43.6[c]	77.0[b,c]	59.8[c]	1.01[b,c]	0.78[c]		
Δ	-17.1[b]	-7.3	-23.5[b]	-10.0	-0.31[b]	-0.14	-23.5[b]	-15.2
Lysolecithin								
Control plasma	2.8	2.9	5.0	5.2	0.10	0.10		
Incubated plasma	6.2[c]	5.7[c]	11.3[c]	10.2[c]	0.22[c]	0.20[c]		
Δ	+3.4	+2.8	+6.3	+5.0	+0.12	+0.10	+120.0	+100.0
Free cholesterol								
Control plasma			30.8[b]	20.2	0.80[b]	0.52		
Incubated plasma			21.6[b,c]	16.6[c]	0.56[b,c]	0.43[c]		
Δ			-9.2[b]	-3.6	-0.24[b]	-0.09	-30.0[b]	-17.2
Cholesteryl ester								
Control plasma	64.0[b]	37.3	143.7[b]	83.8	2.24[b]	1.31		
Incubated plasma	70.6[b,c]	41.0[c]	158.7[b,c]	92.1[c]	2.48[b,c]	1.44[c]		
Δ	+6.6[b]	+3.7	+15.0[b]	+8.3	+0.24[b]	+0.13	+10.2	+9.9

[a]Each value is the mean of eight observations.

[b]Significant difference between values for animals at 35 C and animals at 22 C.

[c]Significant difference between values for incubated plasma and those for control plasma.

silicic acid (100 mesh; A.R. Mallinchrodt Chemical Works, N.Y.) according to the method of Moore and Doran (10). Only the cholesteryl ester, free cholesterol and total phospholipid fractions were collected for further analysis. The purity of these fractions was checked by thin layer chromatography using glass plates coated with Kieselgel G (E. Merck, Darmstadt, Germany). The total phospholipid extracts were further fractionated on thin layer plates coated with Kieselgel G without binder (Camag Ltd., Muttenz, Switzerland); the solvent system was chloroform-methanol-acetic acid-water 25:15:4:2 v/v as described by Skipski et al. (11). The chromatoplates were sprayed with a 0.1% w/v solution of 2,4-dichlorofluorescein in methanol and the lecithin and lysolecithin bands were visualised under UV light. The bands were scraped from the plates and the lecithin and lysolecithin were then eluted with methanol. Identification was by comparison with known standards. The fatty acid compositions of the cholesteryl ester, lecithin and lysolecithin fractions were determined by gas liquid chromatographic methods described in detail by Moore and Williams (12), and the absolute concentrations of these lipid fractions were determined by the addition to each fraction of a known amount of *n*-heptdecanoic acid as an internal standard (13). Free cholesterol was determined by the method of MacIntyre and Ralston (14). Wherever possible, reactions were carried out under nitrogen and 2,6-di-*t*-butyl-*p*-cresol was added to the chromatographic sprays and solvents to minimize autoxidation. Solvents were distilled before use.

RESULTS

Experiment 1

Figure 1 shows the changes which were observed in the concentrations (mg/100 ml plasma) of the total lipids of the plasma when the environmental temperature of the animals was increased from 22 to 35 C. The steady decline observed in the concentration of the total plasma lipids during exposure to the higher environmental temperature resulted in a new equilibrium value for the plasma lipid concentration of ca. 210 mg/100 ml plasma compared to ca. 290 mg/100 ml plasma observed in animals at 22 C. No significant change was detected in the total blood volume (mean, 59.5 ml/kg body wt) of the animals at the different environmental temperatures.

Experiment 2

The concentrations of the lecithin, lysolecithin, free cholesterol and cholesteryl ester fractions of the bovine plasma samples from the animals maintained at 22 C and 35 C, together with the effect of the in vitro incubation for 20 hr at 38 C upon the concentrations of these

fractions, are shown in Table I. Values for the concentrations of the four lipid fractions in terms of milligram lipid per 100 ml plasma and μmol/ml plasma have been obtained using the conversion factors of Christie et al. (13). The concentrations of the lecithin and cholesteryl ester fractions in the plasma when the animals were maintained at 35 C were significantly lower than the corresponding concentrations of these lipid fractions in the plasma when the animals were maintained at 22 C. A similar difference existed between the concentrations of free cholesterol in the plasma at the two temperatures. There was no significant difference in the concentration of the lysolecithin fraction in the plasma of the animals at the different environmental temperatures. Incubation of the plasma resulted in significant increases in the concentrations of the cholesteryl ester and lysolecithin fractions and a significant decrease in the concentration of the lecithin fraction for the animals at both environmental temperatures. When the changes in the concentrations of the plasma lipids are expressed in terms of Δ μmol/ml plasma, there is reasonable agreement within the animals at both environmental temperatures between the increase that occurred in the cholesteryl ester fraction and the decrease that occurred in the lecithin and free cholesterol fractions during incubation. However when the animals were maintained at 22 C the change that occurred in the concentration of the lysolecithin fraction during incubation was smaller than the changes observed for the other three fractions; the possible action of lysophosphatidyl hydrolases in the plasma has been mentioned previously (5).

The fatty acid compositions of the lecithins, lysolecithin and cholesteryl ester fractions in the plasma samples, both before and after incubation, from the animals maintained at 22 C and 35 C are given in Table II. The concentration of 18:2 in the cholesteryl ester fraction was significantly lower and the concentrations of 16:0, 18:0 and 20:3 were significantly higher in the plasma of the animals when they were maintained at 35 C than when they were maintained at 22 C. In the lysolecithin fraction the concentration of 18:0 was significantly lower and the concentrations of 16:1 and 18:3 and 20:3 were significantly higher in the animals at 35 C than at 22 C. There was very little difference between the fatty acid composition of the lecithin fraction for the animals maintained at the two environmental temperatures. The pattern of the fatty acid changes which occurred in the lecithin, lysolecithin and cholesteryl ester fractions of the plasma as a result of incubation were in the

TABLE II

Effect of Incubation for 20 hr at 38 C on Fatty Acid Composition[a] of Lecithin, Lysolecithin and Cholesteryl Ester Fractions of Bovine Plasma from Animals Maintained at 22 C and 35 C

Lipid	16:0		16:1		18:0		18:1		18:2 (*n*-6)		18:3 (*n*-3)		20:3 (*n*-9)		20:4 (*n*-6)	
	22C	35C	22C	35C	22C	35C	22C	35C	22C	35C	22C	35C	22C	35C	22C	35C
Lecithin																
Control plasma	13.4	14.1	0.4[b]	0.7	26.7	26.8	14.1	12.2	30.1	29.1	1.3	1.3	5.7[b]	4.6	8.2	8.5
Incubated plasma	13.1	13.3	0.4	0.6	28.3[c]	27.7[c]	16.0	14.8	25.8[c]	24.5[c]	0.7[c]	1.0	6.6[b,c]	5.8[c]	8.9	9.7
Lysolecithin																
Control plasma	23.6	21.4	1.2[b]	3.4	30.5[b]	21.3	14.9	14.2	19.4	20.0	1.9[b]	3.4	4.5[b]	12.1	3.6	4.0
Incubated plasma	29.4[c]	26.7[c]	0.6	1.9[c]	54.2[b,c]	45.8[c]	7.4[c]	10.1[c]	4.4[c]	6.6[c]	0.7[b,c]	1.5[c]	1.8[b,c]	5.3[c]	1.5[c]	2.1[c]
Cholesteryl ester																
Control plasma	5.4[b]	8.3	1.9	2.0	0.7[b]	2.0	4.7	5.0	73.2[b]	66.6	5.2[b]	6.9	---	---	5.1	5.5
Incubated plasma	5.5[b]	7.7	1.9	2.1	0.7[b]	1.6	4.5	5.1	72.7[b]	66.2	5.4[b]	6.6	---	---	5.7	6.2

[a]Major components, wt % of total. Each value is the mean of eight observations.
[b]Significant difference between values for animals at 35 C and animals at 22 C.
[c]Significant difference between values for incubated plasma and those for control plasma.

TABLE III

Effect of Incubation for 20 hr at 38 C on Absolute Concentrations of Fatty Acids[a] Contained in Lecithin, *Lysolecithin and Cholesteryl Ester Fractions of Bovine Plasma from Animals Maintained at 22 C and 35 C*

Lipid	16:0		16:1		18:0		18:1		18:2 (*n*-6)		18:3 (*n*-3)		20:3 (*n*-9)		20:4 (*n*-6)	
	22C	35C	22C	35C	22C	35C	22C	35C	22C	35C	22C	35C	22C	35C	22C	35C
Lecithin																
Control plasma	9.8[b]	7.4	0.3	0.4	19.5[b]	13.9	10.3[b]	6.3	22.1[b]	14.9	0.9	0.7	4.2[b]	2.4	6.0[b]	4.4
Incubated plasma	7.4[b,c]	6.0[c]	0.2	0.2	15.8[b,c]	12.4[c]	9.0[b]	6.4	14.6[b,c]	11.1[c]	0.4[c]	0.4	3.7[b]	2.5	5.0	4.4
Lysolecithin																
Control plasma	0.6	0.6	0.1	0.1	0.8	0.6	0.4	0.4	0.6	0.6	0.1	0.1	0.1[b]	0.3	0.1	0.1
Incubated plasma	1.8[c]	1.5[c]	0.1	0.1	3.4[b,c]	2.6[c]	0.5	0.6	0.3	0.4	0.1	0.1	0.1[b]	0.3	0.1	0.1
Cholesteryl ester																
Control plasma	3.5	3.0	1.1[b]	0.7	0.4[b]	0.7	3.0[b]	1.7	46.9[b]	24.8	3.3	2.5	---	---	3.3	2.2
Incubated plasma	3.9[b]	3.1	1.3[b]	0.8	0.5	0.6	3.1[b]	2.1	51.4[b,c]	27.3	3.8[b]	2.7	---	---	4.0[b,c]	2.7

[a]mg Fatty acid per 100 ml plasma.
[b]Significant difference between values for animals at 35 C and animals at 22 C.
[c]Significant difference between values for incubated plasma and those for control plasma.

main similar for the animals at both temperatures. In the lecithin fraction of the incubated plasma, the concentration of 18:2 was significantly lower and the concentrations of 18:0 and 20:3 significantly higher than the corresponding values found in the lecithin of the control plasma. The decrease which occurred in the concentration of 18:2 in the lecithin fraction was accompanied in the lysolecithin fraction by a significant increase in the concentrations of 16:0 and 18:0 and a significant decrease in the concentrations of 16:1, 18:1, 18:2, 18:3, 20:3 and 20:4. In the cholesteryl ester fraction, where 18:2 accounted for 73% and 66% of the total fatty acids present, respectively, in the animals at 22 C and 35 C, incubation failed to produce any significant difference in the fatty acid composition.

Table III shows the absolute concentrations (expressed as mg/100 ml plasma) of the individual fatty acids contained in the lecithin, lysolecithin and cholesteryl ester fractions of the plasma samples, both before and after incubation, for the animals, at 22 C and 35 C. The concentrations of 16:0, 18:0, 18:1, 18:2, 20:3 and 20:4 in the lecithin fraction and the concentrations of 16:1, 18:1 and 18:2 in the cholesteryl ester fraction were all significantly lower in the plasma from the animals at 35 C compared with the animals at 22 C. In the cholesteryl ester fraction, the animals at 35 C had significantly higher concentrations of 18:0 than at 22 C. In the lysolecithin fraction the concentration of 20:3 was significantly higher in the animals at 35 C. Incubation resulted in significant decreases in the concentrations of 16:0, 18:0 and 18:2 in the lecithin fraction and increased concentrations of 16:0 and 18:0 in the lysolecithin fraction of the plasma from the animals at both temperatures. In the cholesteryl ester fraction the increase which occurred in the concentrations of 18:2 and 20:4 as a result of incubation was only significant for the animals at 22 C.

DISCUSSION

Evidence is provided by the present results to show that in steers, exposure to a high environmental temperature considerably reduces the concentration of the major circulatory lipid fractions. In the animals from which consecutive daily plasma samples were taken (experiment 1) the change in environmental temperature from 22 to 35 C had no sudden effect on the lipid concentration. Exposure to the higher environmental temperature caused a steady decline in the concentration of the circulatory lipids, reaching a new equilibrium

level after 7-8 days which was ca. 30% below the concentration observed at 22 C. In experiment 2 when the animals were maintained at a temperature of 35 C, the concentrations of the lecithin, free cholesterol and cholesterol ester fractions were reduced to ca. 60% of their corresponding concentrations observed in the animals at 22 C. It is therefore of interest that recent investigations of O'Kelly (15) into seasonal variations in the plasma lipid composition of both Zebu and British type cattle demonstrated that, irrespective of breed, the concentrations of total lipid, cholesterol, phospholipid and triglyceride were all significantly influenced by season and were highest in the winter and lowest in the summer. Although water intake is directly related to ambient temperature and dry matter consumption in cattle, it was concluded that these differences could not be accounted for by any hemodilution effect. The failure to observe any significant change in total blood volume during the present experiment is further direct evidence that the plasma lipid changes observed at the higher environmental temperature were in no way the result of hemodilution but an effect of acclimatization to hyperthermia.

The causative factors involved in the plasma lipid changes which occur during exposure to a high environmental temperature are not known. However evidence exists to show that exposure to elevated temperatures may exert influences on other aspects of lipid metabolism in the animal, which in turn would affect the composition and metabolism of the circulatory lipids. Thus the work of Bobek and Ginter (16), in which rats were exposed to a temperature of 35 C for a prolonged period, resulted in a decrease in the concentration of cholesterol in various tissues, a decrease in the synthesis of cholesterol and fatty acids from acetate-1-^{14}C in the liver and a decrease in the concentration of esterified fatty acids in the plasma. Bishop et al. (17) have also shown that in rats maintained at 35-40 C there was a decreased incorporation of acetate-1-^{14}C into the free cholesterol of the liver when compared with rats maintained at 24-28 C. With respect to ruminants, a significant decrease in total volatile fatty acid production, in particular acetic acid production, has been shown to occur in cattle under increased ambient temperature (18,19). Therefore sufficient data exists to indicate that exposure to high environmental temperatures may have pronounced effects on lipid metabolism in general.

The present investigations clearly demonstrate that under conditions of high environmental temperatures there is a large decrease in the concentration of 18:2 circulating in the lecithin and cholesteryl ester fractions (Table III). In the lecithin fraction there was no difference in the percentage fatty acid composition between the animals at the two environmental temperatures, and the decrease in the absolute concentration of circulatory 18:2 could be accounted for entirely by the decrease that occurred in the concentration of the plasma lecithin. However, wereas 18:2 constituted at least 72% of the total fatty acids present in the cholesteryl ester fraction of the plasma when the animals were maintained at 22 C, the proportion of 18:2 in the cholesteryl ester fraction was reduced to ca. 66% of the total fatty acid present when the animals were maintained at 35 C. Recently the work of Kuiper et al (20) on the physical and biochemical changes that occur in erythrocytes from hamsters has also shown that high environmental temperatures affect the content of C_{18} polyunsaturated fatty acids in the erythrocytes. During heat exposure, the concentration of 18:3 in all the major phospholipid fractions present was decreased and the conccentration of 16:0 increased; in the sphingomyelin and phosphatidyl ethanolamine fractions, the concentration of 18:2 was also reduced (20). An increased proportion of saturated fatty acids in depot fats from animals exposed to high environmental temperatures has been observed by several workers and as yet remains unexplained (16,21,22).

It has been established that the LCAT reaction is an important source of the plasma cholesteryl esters in both the nonruminant and ruminant animal (5,23). Recently O'Kelly (24), investigating the plasma lipid composition of genetically different breeds of cattle, has demonstrated an increase in the free-esterified cholesterol ratio under conditions of hyperthermia. It was suggested that the elevated ratio in the heat exposed animals, which resulted mainly from a reduction in the concentration of the esterified cholesterol fraction of the plasma, may have been the result of a hyperthermic effect on the plasma LCAT activity. In the present experiment the mean net esterification of cholesterol after the incubation for 20 hr of plasma from the animals maintained at 22 C was similar to that found previously for steers (5). Maintaining the animals at 35 C resulted in a considerable decrease in the net esterification of cholesterol, compared with the animals maintained at 22 C (Table I). These results do not necessarily indicate that the activity of the LCAT enzyme was lower in the plasma of the animals maintained at 35 C, since the differences in extent of esterification could have been caused by differences in the amount of

lipoprotein substrate present. When the animals were exposed to the high environmental temperature, the concentration of free cholesterol available for esterification in the plasma was significantly reduced, and it has been demonstrated by Monger and Nestel (25) that in human plasma a significant correlation exists between the plasma cholesterol concentration and the rate of esterification. The possibility that the availability of substrate may be the limiting factor in the cholesterol esterification by the LCAT system in the heat exposed animals is, however, challenged when the values for net esterification are expressed in terms of the per cent change that occurred in the various plasma lipid fractions (Table I). For both lecithin and free cholesterol, the percentage change observed in the animals when maintained at 35 C was considerably less than for the animals at 22 C. However the percentage change that occurred in the cholesteryl ester fraction was identical for the animals at both environmental temperatures. It may be possible, therefore, that the limiting factor in the extent of cholesterol esterification observed in the heat exposed animals was not the limited concentration of substrate, but involved the accumulation and accommodation of the product. A concept of a requirement for a "substrate" and "product" lipoprotein in the acyl transferase reaction has been suggested by Glomset (23).

The changes in the fatty acid composition of the lecithin, lysolecithin and cholesteryl ester fractions of the plasma obtained in the present experiment as a result of incubation at 38 C are similar to those already reported for bovine plasma (5). In the present experiment where 18:2 constituted such a high proportion of the total fatty acids present in the plasma cholesteryl esters, the plasma esterification process demonstrated a high specificity for the 18:2 of the lecithin. With the importance of the LCAT system established in the formation of bovine plasma cholesteryl esters, a significant reduction in the acyl transferase reaction in the plasma, for whatever reason, could well account for the significant decrease observed during heat exposure in the concentration and transfer of 18:2 found in the cholesteryl ester fraction (Tables II-III). Recently it has been suggested by Mickel et al. (26) that the primary product of the acyl transferase reaction, namely cholesterol linoleate, is required for erythropoiesis. Decreased availability of cholesterol linoleate might therefore decrease hematopoietic function of depriving the tissues of essential fatty acids, and this may well occur through a reduction in the LCAT activity of the plasma. It is therefore of interest that the work of Kuiper et al. (20) has recently drawn attention to the increase in red cell fragility which occurs when animals are exposed to high environmental temperatures. Furthermore it has also been shown that a deficiency of polyunsaturated fatty acids in the diet results in increased cell fragility (27).

Thus, from the evidence available, it would appear possible that under conditions of high environmental temperatures the integral relationship which exists between the LCAT activity of the plasma and the polyunsaturated fatty acids of the circulation may be seriously disturbed; consequential effects upon other aspects of the lipid metabolism of the animal may also occur.

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REFERENCES

1. O'Kelly, J.C., Austral. J. Biol. Sci. 21:1013 (1968).
2. Bianca, W., J. Dairy Sci. 32:291 (1965).
3. Diven, R.H., H.M. Page, E.S. Erwin and C.B. Roubicek, Amer. J. Physiol. 195:88 (1958).
4. Duncan, W.R.H., and G.A. Garton, J. Lipid Res. 3:53 (1962).
5. Noble, R.C., J.C. O'Kelly and J.H. Moore, Biochim. Biophys. Acta 270:519 (1972).
6. Campbell, T.J., B. Frohman and B.R. Reeve, J. Lab. Clin. Med. 5:768 (1958).
7. Nitshe, G.A., and P.P. Cohen, J. Clin. Path. 17:239 (1947).
8. Sperry, W.M., J. Biol. Chem. 111:467 (1935).
9. Nelson, G.J., and N.K. Freeman, Ibid. 234, 1375-1380 (1959).
10. Moore, J.H., and B.M. Doran, Biochem. J. 84:506 (1962).
11. Skipski, V.P., R.F. Peterson and M. Barclay, Ibid. 90:374 (1964).
12. Moore, J.H., and D.L. Williams, Biochim. Biophys. Acta 84:41 (1964).
13. Christie, W.W., R.c. Noble and J.H. Moore, Analyst 95:940 (1970).
14. MacIntyre, I., and M. Ralston, Biochem. J. 56:XLIII (1954).
15. O'Kelly, J.C., Comp. Biochem. Physiol. 43:283 (1972).
16. Bobek, P., and E. Ginter, J. Nutr. 89:373 (1966).
17. Bishop, J.A., C.E. Cornelius and S. Abraham, Cornell Vet. 55:500 (1965).
18. Weldy, J.R., R.E. McDowell, P.J. Van Soest and J. Bond, J. Anim. Sci. 23:147 (1964).
19. Kelley, R.O., F.A. Martz and H.D. Johnson, J. Dairy Sci. 50:531 (1967).
20. Kuiper, P.J.C., A. Livne and M. Meyerstein, Biochim. Biophys. Acta 248:300 (1971).
21. Young, D.R., and S.F. Cook, Amer. J. Physiol. 181:72 (1955).
22. Mefford, R.B., M.A. Nyman and W.W. Webster,

Ibid. 195:744 (1958).
23. Glomset, J.A., J. Lipid Res. 9:155 (1968).
24. O'Kelly, J.C., Comp. Biochem. Physiol. 44:313 (1973).
25. Monger, E.A., and P.J. Nestel, Clin. Chim. Acta 15:269 (1967).
26. Mickel, H.S., E.L. Foulds, D.A. Clark and E.C. Larkin, Lipids 6:740 (1971).
27. MacMillan, A.L., and H.M. Sinclair, in "Essential Fatty Acids," Edited by H.M. Sinclair, London, Butterworths Scientific Publications, 1958, p. 208.

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Studies on Metabolism of Linoleic-1-^{14}C Acid in Testes of Hypophysectomized Rats

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ABSTRACT

Studies are reported on the influence of hypophysectomy and pituitary gonadotrophins on the interconversion and incorporation of linoleic-1-^{14}C acid into lipid classes of mature rat testis. Linoleic 1-^{14}C acid was injected into the testes of mature rats 3 weeks after hypophysectomy. Groups of animals were killed at 0.25, 0.50, 1, 3, 12, 24, 36 and 48 hr after injection of the radioactive linoleic acid, and the incorporation of radioactivity into the fatty acids and lipid classes of the testicular lipids was determined. Similar experiments were carried out with hypophysectomized animals injected intramuscularly with luteinizing or follicle stimulating hormones, or both. The specific activities of the triglycerides and phospholipids of the testicular lipid increased to a maximum ca. 1 hr after the injection of the radioactive linoleic acid, then decreased sharply. The general pattern of changes in the specific activities of these compounds indicated that the rate of fatty acid catabolism was greatly increased by hypophysectomy. In contrast, the specific activities of the cholesteryl esters and glyceryl ether diesters changed slowly after reaching a maximum at approximately the same time. This pattern, their accumulation in the lipid, and an increase in the constituent 22:5 indicated that the turnover of these compounds was impaired by hypophysectomy. The conversion of linoleic acid to arachidonic acid was also impaired by hypophysectomy, as evidenced by an increase in the percentage and the high specific activity of 20:3 compared to 20:4. The administration of gonadotrophins partially prevented the effects of hypophysectomy, indicating that some of the enzymes in the testes involved in the metabolism of the lipids are hormone sensitive.

INTRODUCTION

Although the influence of hormones on lipogenesis and mobilization of fatty acids has been studied extensively (1-5), the extent to which hormonal action is involved in the control of the metabolism of cholesterol, triglycerides and phospholipids is not clear. Marked changes have been observed in the testicular lipids of hypophysectomized rats by histochemical (6-9) and chemical studies (10-12), indicating that the hypophyseal hormones play an important role in the metabolism of testicular lipids. Goswami and Williams (13) reported that follicle stimulating (FSH) and luteinizing (LH) hormones, alone or in combination, stimulated the synthesis of polyunsaturated fatty acids, but had little effect on the incorporation of fatty acids into testicular lipids of mature hypophysectomized rats. In further studies, Goswami et al. (14) attributed the effect on the synthesis of polyunsaturated fatty acids to a stimulation of RNA synthesis by gonadotrophins.

Common pathways for the net synthesis of glycerides and glycerophosphatides have been well demonstrated (15-18), and it has been shown that the acyl chains and skeletal moieties of these compounds turn over independently of each other (19-21). Acyl transferase reactions are important in these reactions, but the precise role of these enzyme systems in the synthesis of triglycerides and glycerophosphatides of specific structures has not been elucidated (22-25).

In previous work (26,27), we studied the incorporation of radioactive linoleic acid among the lipid classes in the testes of rats and demonstrated the importance of the fatty acid pool in these reactions. As a prelude to studies of the importance of hormonal control in acyl transferase reactions in the synthesis of phospholipids and triglycerides in the testis, the metabolism of linoleic acid in the testes of hypophysectomized rats was investigated. The results of these studies are presented here.

MATERIALS AND METHODS

Methods for the quantitative analysis of the lipid classes, determination of fatty acid composition (gas liquid chromatography [GLC] of fatty acid methyl esters) and measurements of radioactivity have been described in detail in previous communications (11,26,27). In the present study, linoleic-1-^{14}C acid was obtained from Tracerlab, Inc., Waltham, Mass., meth-

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TABLE I

Per Cent Distribution of Radioactivity in Testicular Lipid of Mature Hypophysectomized Rats Injected Intratesticularly with Linoleic-1-^{14}C Acid 3 Weeks after Hypophysectomy[a]

	Time period, hr							
	.25	.50	1	3	12	24	36	48
	Testis weight, g							
	0.38	0.38	0.43	0.37	0.31	0.28	0.39	0.29
	Lipid, %							
	5.6	5.4	4.3	4.8	5.3	5.8	4.3	6.5
	Radioactivity recovery, %							
Lipid	62.7	59.8	57.9	40.9	29.4	25.6	23.2	18.6
Neutral lipid, % distribution of radioactivity								
Total neutral lipid[b]	71.2	66.3	62.0	59.5	58.1	54.0	55.9	68.7
CE	1.2	2.7	2.5	4.4	6.7	5.5	7.3	12.7
GEDE	3.3	3.0	2.9	4.2	5.9	4.8	6.1	8.4
TG	8.4	12.1	16.4	20.7	22.8	22.6	19.9	28.6
FA	54.7	44.8	34.9	25.8	18.9	16.9	18.4	15.0
DG	2.5	2.8	2.9	3.0	2.5	2.7	2.7	2.7
Polar lipid, % distribution of radioactivity								
Total polar lipid[b]	28.8	33.7	38.5	40.5	41.9	46.1	44.1	31.3
DPG	0.7	1.2	2.1	1.6	1.8	1.6	1.9	1.7
PE	1.7	3.4	4.3	5.0	6.8	7.7	7.1	6.0
PI+PS	2.5	2.6	2.2	2.6	3.5	3.8	4.4	3.1
PC	13.7	18.3	22.8	25.8	27.1	29.3	27.1	16.6
Sph.	0.9	0.7	1.1	3.0	1.6	1.8	1.5	1.6
Lyso Pc	1.5	1.3	4.9	2.1	1.1	1.1	1.2	0.8

[a]Average values from two animals. Abbreviations: CE = cholesteryl esters; GEDE = glyceryl ether diesters; TG = triglycerides; FA = fatty acids; DG = diglycerides; DPG = diphosphatidyl glycerol; PE = phosphatidyl ethanolamine; PI = phosphatidyl inositol; PS = phosphatidyl serine; SPH = sphingomyelin; Lyso-PC = lysophosphatidyl choline.

[b]Determined by difference between radioactivity of total PL or total NL and total radioactivity of sample analyzed.

ylated with diazomethane and purified by thin layer chromatography (TLC) with plates coated with silicic acid impregnated with silver nitrate. GLC and measurement of the radioactivity of the separated components 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 mC/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 injection, had an activity of 0.77 μC and contained 0.128 mg linoleic acid.

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 a dioxane-water solution described by Snyder (28). The latter solution was used for the analysis of material recovered from chromatoplates used in TLC. Counting efficiency for carbon 14 was 85% in toluene and 71% in dioxane-water solution. Determination of percentage distribution of radioactivity and specific activities of lipid classes and fatty acids was carried out as described in previous work (26). In this procedure, four different solvent systems are used to separate the lipid classes and provide duplicate checks on all components. Recovery of radioactivity in known components of the lipid classes was generally 90-95%. The total polar lipid fraction and total neutral lipid fraction separate at the origin and the front, respectively, in two of these solvent systems. The percentage distribution of radioactivity in these fractions is determined by the difference between that applied to the plate, and that analyzed in the separated PL or NL fractions.

Animals

Hypophysectomized and normal male rats of the Sprague-Dawley strain, 200-255 g body wt,

TABLE II

Composition of Long Chain Fatty Acids of Lipid Classes of Testicular Lipids of Mature Rats 3 Weeks after Hypophysectomy (wt %)

	Lipid, wt %									
	Cholesteryl esters		Glyceryl ether diesters		Triglycerides		Phosphatidyl choline		Phosphatidyl ethanolamine	
Fatty acid[a]	N[b] 0.49	H[b] 8.6	N 0.31	H 3.5	N 4.5	H 8.7	N 29.3	H 22.9	N 27.2	H 17.1
16:0	14.5	6.0	19.2	9.5	28.6	19.7	57.1	42.6	29.6	23.9
18:0	5.8	3.0	7.8	5.8	4.2	12.1	4.3	4.9	11.7	13.3
18:1	13.5	6.5	9.0	5.9	10.4	6.8	12.9	21.7	6.6	8.6
18:2	9.1	1.3	2.8	1.3	2.7	4.3	3.0	1.4	2.8	1.4
20:3	3.7	4.7	2.5	4.3	1.7	3.2	1.1	2.1	0.6	1.1
20:4	14.1	5.8	6.3	3.6	3.9	2.6	7.7	9.6	10.9	22.1
22:4	3.1	2.1	5.2	7.7	5.7	3.6	0.5	0.8	5.5	3.2
22:5	12.0	51.8	15.6	38.5	32.3	30.3	9.4	6.5	28.9	22.9

[a]Shorthand designation for fatty acids; number before colon = chain length; number after colon = number of double bonds.

[b]N = animals of normal group; H = animals of hypophysectomized group.

were obtained from the Hormone Assay Lab., Chicago. The animals were housed in individual cages and fed ad libitum a semisynthetic diet containing vitamins and minerals in the required amounts (12), to which was added 10% safflower seed oil.

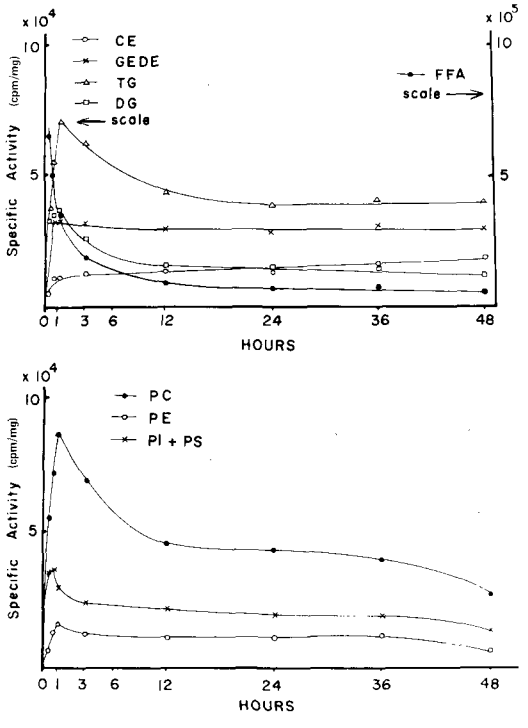

FIG. 1. Time course changes in the specific activity of the lipid classes of the testicular lipids of mature hypophysectomized rats injected intratesticularly with linoleic-1-^{14}C acid 3 weeks after hypophysectomy. TG = triglyceride, DG = diglyceride, FFA = free fatty acid, GEDE = glyceryl ether diesters, CE = cholesteryl esters, PC = phosphatidyl choline, PE = phosphatidyl ethanolamine, PI = phosphatidyl inositol, PS = phosphatidyl serine.

In the first experiment, animals were killed in pairs 3 weeks after hypophysectomy by withdrawal of blood from the aortas at 0.25, 0.50, 1, 3, 12, 24, 36 and 48 hr after injection of radioactive linoleic acid. The testes of the animals were excised, frozen on dry ice and stored at -20 C. Prior to extraction of the lipid, each testis was allowed to thaw on the surface at room temperature to facilitate decapsulation and weighed. After each testis was weighed, the lipid was extracted with chloroform-methanol 2:1 and the distribution of radioactivity among the lipid classes determined. Changes in the specific activities of the lipid classes and their constituent fatty acids over the 48 hr period were also followed. In an accessory experiment, the percentage distribution of radioactivity in the fatty acids of the testicular lipids of groups of four to six hypophysectomized and normal animals was compared at 3, 12 and 24 hr after injection of radioactive linoleic-1-^{14}C acid.

In another experiment, 24 hypophysectomized rats were divided into four groups and injected intramuscularly biweekly for 3 weeks with 0.5 ml saline or 0.5 ml saline containing 0.5 mg LH, (NIH-LH-S11), 0.5 mg FSH, (NIH-FSH-S3), or 0.25 mg FSH and 0.25 mg LH. At the end of 3 weeks, 12 hr after the last hormone injection, the animals were injected intratesticularly with 50 μl of the linoleic-1-^{14}C acid emulsion and sacrificed 3 hr later by withdrawal of blood from the aortas. The testes of these animals were also excised, frozen on dry ice, stored at -20 C and then decapsulated (after partial thawing) and weighed. After the testes were weighed, the lipid was extracted

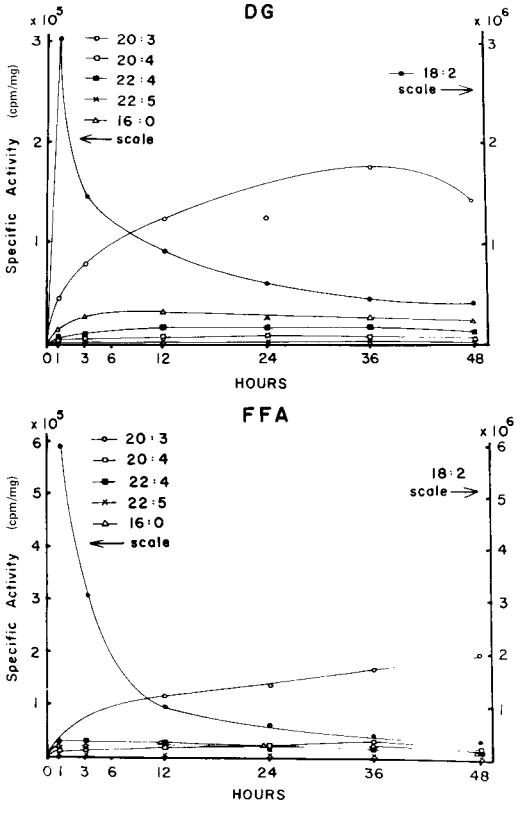

FIG. 2. Time course changes in specific activities of the fatty acids of diglycerides (DG) and free fatty acids (FFA) in testicular lipids of mature hypophysectomized rats injected with linoleic-1-^{14}C acid 3 weeks after hypophysectomy. Shorthand designation is used to denote fatty acids; number before colon = chain length, number after colon = number of double bonds.

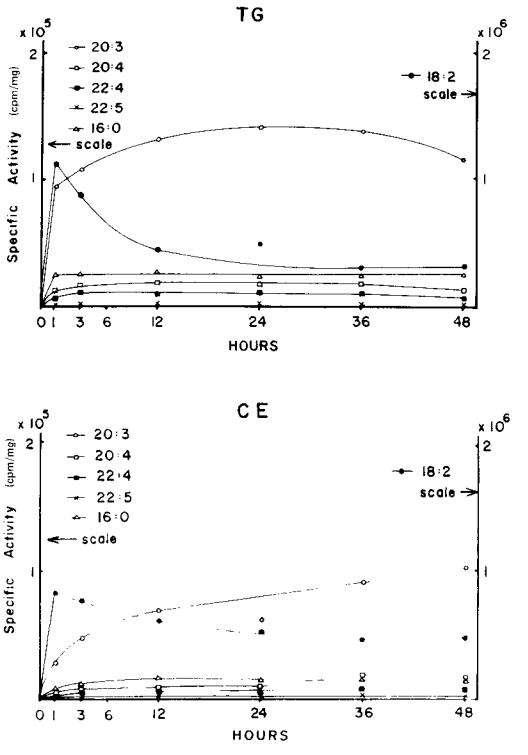

FIG. 3. Time course changes in specific activities of the fatty acids of triglycerides (TG) and cholesteryl esters (CE) of the testicular lipids of mature hypophysectomized rats injected with linoleic-1-^{14}C acid 3 weeks after hypophysectomy. Shorthand designation is used to denote fatty acids; number before colon = chain length, number after colon = number of double bonds.

with chloroform-methanol 2:1 and recovered for analysis as in the first experiment.

RESULTS

Data on the recovery and percentage of radioactivity among the lipid classes of the testes of the hypophysectomized animals over the 48 hr period, following the injection of linoleic-1-^{14}C acid in the first experiment, are summarized in Table I. At the end of 48 hr, ca. 18% of the injected radioactivity remained in the lipid of the testis. The percentage distribution of radioactivity among the lipid classes indicated that the turnover of these compounds was different in hypophysectomized and normal animals, previously reported (26). The major difference was in the larger proportion of radioactivity that accumulated in the neutral lipids in the hypophysectomized animals. In normal animals, ca. 35% of the radioactivity was distributed in the neutral lipids and 65% in the phospholipids. This ratio was reversed by hypophysectomy, as illustrated in Table I. These changes were produced largely by a gradual accumulation of the radioactivity in the cholesteryl esters, glyceryl ether diesters and triglycerides. This was presumably caused by a slower turnover of these compounds compared to the phospholipids in the hypophysectomized animals. These observations are in accord with the effect of hypophysectomy on the composition of the lipid as observed previously (11) and as illustrated in Table II, which shows that the percentage of cholesteryl esters, glyceryl ether diesters and triglycerides are increased at the expense of the phospholipids.

Changes in the specific activities of the lipid classes during the metabolism of the injected linoleic-1-^{14}C acid are shown in Figure 1. The injected linoleic acid was incorporated rapidly into the lipid, as evidenced by the rapid decrease of the specific activity of the free fatty acid fraction and simultaneous increases in the

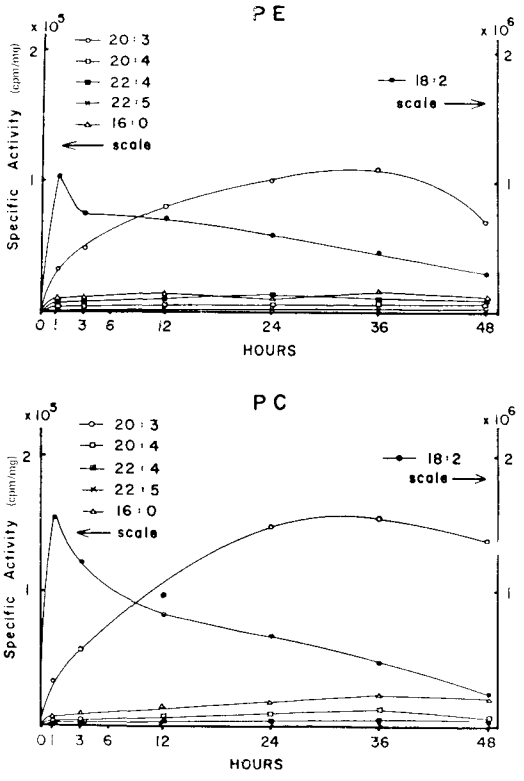

FIG. 4. Time course changes in the specific activities of the fatty acids of phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) of the testicular lipids of mature hypophysectomized rats injected with linoleic-1-^{14}C acid 3 weeks after hypophysectomy. Shorthand designation is used to denote fatty acids; number before colon = chain length, number after colon = number of double bonds.

specific activity of PC, DG and TG. The peak in the specific activity of these compounds, PC, PI and PS was reached in ca. 1 hr. This pattern was much different from that obtained with normal animals (26) and also indicated that the metabolism of these compounds was influenced by hypophysectomy. The maximum specific activity of the glyceryl ether diesters and cholesteryl esters also occurred at ca. 1 hr after injection of the radioactive linoleic acid, but remained high indicating a slow turnover rate for these compounds.

Hypophysectomy also produced a marked effect on the fatty acid composition of the lipid classes, as illustrated in Table II. Changes in the fatty acid composition varied in the different lipid classes. Noteworthy was the relatively large increase in the percentage of 22:5 in the cholesteryl esters and glyceryl ether diesters. Changes in fatty acid composition also occurred in TG, PC and PE, but in different ways except for palmitic acid which decreased in all of the lipid classes.

The time course changes in specific activity of the fatty acids (Fig. 2-4) also demonstrated the marked effects of hypophysectomy on fatty acid metabolism. The pattern of the changes in specific activities was remarkably similar in all of the lipid classes. The specific activity of linoleic acid decreased rapidly as it was catabolized and converted to long chain polyunsaturated fatty acids. Radioactivity was incorporated into palmitic acid by endogenous synthesis from radioactive acetate produced in the catabolism of linoleic-1-^{14}C acid. Some radioactivity appeared to be incorporated into stearic and oleic acids, but a precise analysis of the distribution of radioactivity in these fatty acids could not be made. The high specific activity of 20:3, which is an intermediate in the conversion of linoleic acid to arachidonic acid,

TABLE III

Per Cent Distribution of Radioactivity in Total Fatty Acids of Testicular Lipids of Hypophysectomized[a] and Normal Mature Rats after Intratesticular Injection of Linoleic-1-^{14}C Acid

Fatty acid	3rd hr		12th hr		24th hr	
	N[b]	H[b]	N	H	N	H
	5 animals	6 animals	6 animals	5 animals	6 animals	4 animals
16:0[c]	3.4 ± 0.2[d]	9.0 ± 0.9	6.0 ± 0.2	15.5 ± 0.6	11.2 ± 1.1	12.8 ± 0.7
18:2	75.2 ± 0.5	67.6 ± 1.1	60.1 ± 0.9	54.2 ± 1.1	42.7 ± 1.8	47.5 ± 1.3
20:3	4.1 ± 0.4	6.1 ± 0.2	5.8 ± 0.2	9.6 ± 0.6	5.9 ± 0.3	12.4 ± 0.8
20:4	8.5 ± 0.3	1.4 ± 0.2	12.8 ± 0.5	2.7 ± 0.2	17.0 ± 1.1	3.5 ± 0.5
22:4	0.9 ± 0.3	0.8 ± 0.1	1.6 ± 0.1	0.9 ± 0.1	3.2 ± 0.3	1.4 ± 0.1
22:5	0.6 ± 0.1	0.5 ± 0.03	0.9 ± 0.1	0.7 ± 0.1	2.3 ± 0.3	1.4 ± 0.2

[a]Three weeks after hypophysectomy.

[b]N = normal group; H = hypophysectomized group.

[c]Shorthand designation for fatty acids. Number before colon = chain length; number after colon = number of double bonds.

[d]M ± SE.

showed that it was readily formed but was converted to arachidonic acid and 22:5 at a much slower rate than its formation. In accord with this observation, the data in Table II indicated that 20:3 accumulated to a higher level in hypophysectomized than normal animals. The effect of hypophysectomy on the metabolism of the fatty acids was also indicated by data on the percentage distribution of the radioactivity among individual fatty acids. These data (Table III) showed that the percentage distribution of radioactivity increased faster and to a higher level in palmitic acid, as well as 20:3 in the hypophysectomized animals. In contrast, the percentage of the radioactivity accumulating in the 20:4, 22:4 and 22:5 was considerably lower in the hypophysectomized than the normal animals. These data, together with those in Figures 2-4, demonstrated that the conversion of 20:3 was impaired by hypophysectomy.

Effects of the intramuscular injections of LH and FSH into hypophysectomized animals are illustrated in Table IV. The hormone injections did not influence the effect of hypophysectomy on growth of the animals. However the weights of the testes of these animals were almost double those of the animals in the control group, and the percentage of the testes of the body weight was almost the same as that of the animals in the normal group. The hormone injections also had an effect on the composition of lipid classes, as illustrated in Table IV. Although the composition of the lipid classes was far from normal, the hormone injections mediated the changes produced by hypophysectomy. There were some differences between the effect of LH and FSH; LH appeared to be more generally active. The hormone injections also maintained the percentage of the 22:5 acid and the ratio of the linoleic acid (18:2) to the 20:3 acids near normal, as illustrated in Table V. These results showed that the percentage of 22:5 was reduced to near normal and the ratio of 18:2 to 20:3, which was ca. 1-2 in the control hypophysectomized animals, was almost normal (5:1) in the animals treated with the hormones, especially LH. The effect of the hormone injections on fatty acid composition was also demonstrated by the distribution of radioactivity, as illustrated in Table VI. These results showed that the distribution of radioactivity between the 20:3 and 20:4 acids in the hypophysectomized animals was maintained near normal by the injection of LH and FSH, particularly FSH.

DISCUSSION

The present study demonstrates that the

TABLE IV

Organ Weights and Testicular Lipid Composition of Normal and Hypophysectomized Rats Treated with LH, FSH and LH + FSH[a]

Weights and lipids	Normal	Hypophysectomy			
		Control	LH	FHS	LH + FSH
	5 animals	6 animals	4 animals	6 animals	6 animals
Original wt, g	249 ± 3.4[b]	226 ± 4.6	233 ± 1.4	235 ± 3.8	241 ± 2.0
Final wt, g	340 ± 4.5	225 ± 4.4	240 ± 5.4	231 ± 3.3	250 ± 3.4
Testis wt, g	1.57 ± 0.06	0.54 ± 0.01	0.96 ± 0.08	0.91 ± 0.07	0.96 ± 0.09
Lipid, mg	48.0 ± 3.3	22.1 ± 0.8	32.5 ± 1.7	37.2 ± 2.2	36.0 ± 3.8
Lipid, %	3.0 ± 0.1	4.1 ± 0.2	3.4 ± 0.1	3.8 ± 0.3	3.7 ± 0.1
Tissue, mg/100 g					
CE	14 ± 0.7	354 ± 26	126 ± 32	173 ± 33	132 ± 33
GEDE	9 ± 0.7	142 ± 18	34 ± 11	76 ± 11	52 ± 14
TG	142 ± 12	346 ± 11	203 ± 13	336 ± 32	311 ± 47
FA	46 ± 5	140 ± 15	103 ± 3	98 ± 8	107 ± 12
C	185 ± 6	396 ± 23	324 ± 27	380 ± 31	308 ± 17

[a]Animals injected intramuscularly biweekly for 3 weeks after hypophysectomy when they were sacrificed. Abbreviations: LH = luteinizing hormone; FSH = follicle stimulating hormone.

[b]M ± SE.

TABLE V

Effect of Gonadotrophins (LH, FSH and LH + FSH) on Fatty Acid Composition of Testicular Lipid of Hypophysectomized Rats (wt %)[a]

Fatty acid[b]	Normal	Hypophysectomy			
		Control	LH	FSH	LH + FSH
16:0	32.8	15.3	21.4	12.5	23.6
18:0	7.4	7.5	7.5	8.2	7.8
18:1	10.4	9.9	10.6	13.3	12.4
18:2	5.0	2.1	5.3	4.1	6.2
20:3	1.2	3.7	1.8	2.7	2.3
20:4	14.5	14.9	14.3	17.3	15.4
22:4	2.5	3.8	1.9	3.7	2.9
22:5	20.9	36.6	22.4	26.8	21.2

[a]Mature animals injected biweekly for 3 weeks after hypophysectomy when they were sacrificed. For abbreviations see Table IV.

[b]Shorthand designation for fatty acids; number before colon = chain length; number after colon = number of double bonds.

metabolism of testicular lipids is markedly influenced by hypophysectomy. Some of these effects may arise indirectly as a result of atrophy of the testes. However there appears to be little doubt that the hypophysis plays an important role in the regulation of the metabolism of testicular lipids. A most important factor relative to the composition of the lipid appears to be the high rate of catabolism of the fatty acids as indicated by the shape of the time course curves of the specific activities of the TG, PC, PE and PI+PS. This factor and differences in rates of turnover of the lipid classes undoubtedly account to a large extent for the effects of hypophysectomy on lipid class composition of the testicular lipids. The maximum specific activities of these lipids in the testes of normal animals did not occur until ca. 24 hr after intratesticular injection of radioactive linoleic acid (26). The sharp maximum in the specific activity curves, very soon after injection of the radioactive linoleic acid in the testes of the hypophysectomized animals, appears to be caused by dominance of the catabolic process.

All of the radioactivity in the palmitic acid must arise by endogenous synthesis from radioactive acetate derived from the catabolism of linoleic-1-^{14}C acid. Hence the more rapid decrease in the specific activity of the linoleic acid, together with a greater and more rapid accumulation of radioactivity in palmitic acid in the hypophysectomized animals, also indicates that the rate of catabolism is increased by hypophysectomy. The large decrease in the percentage of palmitic acid indicates that the catabolism of this acid is also increased by hypophysectomy. The catabolism (per se) of

TABLE VI

Per Cent Distribution of Radioactivity in Fatty Acids of Testicular Lipid of Normal and Hypophysectomized Rats, Treated with LH, FSH and LH + FSH, 3 hr after Intratesticular Injection of Linoleic-1-^{14}C Acid

Fatty acid	Normal	Hypophysectomy			
		Control	LH	FSH	LH + FSH
	5 animals	6 animals	4 animals	6 animals	6 animals
16:0[b]	3.4 ± 0.2[c]	9.0 ± 0.9	4.7 ± 0.4	8.4 ± 1.0	5.9 ± 0.9
18:2	75.2 ± 0.5	67.6 ± 1.1	73.6 ± 0.6	66.2 ± 1.4	68.0 ± 2.0
20:3	4.1 ± 0.4	6.1 ± 0.2	4.2 ± 0.1	5.9 ± 0.4	5.3 ± 0.4
20:4	8.5 ± 0.3	1.4 ± 0.2	2.8 ± 0.3	4.0 ± 0.4	4.6 ± 0.2
22:4	0.9 ± 0.3	0.8 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
22:5	0.6 ± 0.1	0.5 ± 0.03	0.5 ± 0.1	0.7 ± 0.1	0.7 ± 0.1

[a]Mature animals injected biweekly for 3 weeks after hypophysectomy when they were sacrificed. For abbreviations see Table IV.

[b]Shorthand designation for fatty acids. Number before colon = chain length; number after colon = number of double bonds.

[c]M ± SE.

other fatty acids might also be increased, but could not be determined in the present study. The increase in the percentage of 22:5 in the total lipid may be attributed to its accumulation in cholesteryl esters and glyceryl ether diesters because of their slow turnover and because of its slow release or participation in transferase reactions compared to other fatty acids. That the rate of transformation or selectivity, or both, may exist among individual fatty acids in transferase reactions (in vivo) is well demonstrated by in vitro experiments with acyl transferase systems (24,25).

Examination of the effect of hypophysectomy on fatty acid composition shows that, except for palmitic acid, the individual fatty acids are decreased in some lipid classes and increased in others. Changes in fatty acid composition can occur by effects on the enzyme systems involved in their synthesis and their release, or transferase reactions among the lipid classes and the fatty acid pool from which they are catabolized. In order to define the effects on these reactions more precisely, tracer experiments of the type reported here will have to be carried out with each individual fatty acid.

The realtively high specific activity of the 20:3 and the increase in its percentage indicate that its conversion to arachidonic acid and 22:5 is impaired by hypophysectomy. These observations also indicate that separate enzyme systems are involved in the conversion of 18:2 to 20:3 and 20:3 to higher polyunsaturated acids. These enzyme systems appear to be hormone sensitive, as evidenced by the effect of injections of LH and FSH on the relative proportions of 20:3, 20:4, 22:4 and particularly 22:5. This effect also was demonstrated by the distribution of radioactivity between 20:3 and 20:4 in the hormone-treated animals. The ratio of the distribution of radioactivity in 20:3 to 20:4 in the testicular lipids of normal animals was ca. 1:2 compared to 5:1 in the hypophysectomized animals. In the hypophysectomized animals treated with LH and FSH, the ratio of these acids was ca. 1:0.9. These observations also indicate that the hypophysis has an important role in the interconversion of polyunsaturated fatty acids.

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REFERENCES

1. Rudmann, D., M.D. Girolamo, M. Markin and L.A. Garcia, in "The Handbook of Physiology, Adipose Tissues," Edited by A. Renold and C.F. Cahil, Jr., Amer. Physiological Soc., Washington, 1965, p. 533.
2. Dole, V.P., N.Y. Acad. Med. 34:21 (1958).
3. White, J.E., and F.L. Engel, Proc. Soc. Exp. Biol. Med. 99:375 (1958).
4. Vaughan, M., J. Lipid Res. 2:293 (1961).
5. Hollenberg, C.H., M.S. Raben and E.B. Astood, Endocrinology 68:589 (1961).
6. Coombs, J.R., and A.J. Marshall, J. Endocrinol. 13:107 (1956).
7. Steinberger, E., and W.O. Nelson, Endocrinology 59:429 (1955).
8. Niemi, M., and M. Ikonen, Ibid. 70:167 (1962).
9. Smith, P.E., Amer. J. Anat. 45:205 (1930).
10. Gambal, D., and R.J. Ackerman, Endocrinology 80:231 (1967).
11. Nakamura, M., B. Jensen and O.S. Privett, Ibid. 82:137 (1968).
12. Jensen, B., M. Nakamura and O.S. Privett, J. Nutr. 95:406 (1968).
13. Goswami, A., and W.L. Williams, Biochem. J. 105:537 (1967).
14. Goswami, A., J.K. Skipper and W.L. Williams, Ibid. 108:147 (1968).
15. Bremer, J., P.H. Figard and D.M. Greenberg, Biochem. Biophys. Acta 43:477 (1960).
16. Gibson, K.D., J.D. Wilson and S. Udemfriend, J. Biol. Chem. 236:673 (1961).
17. Kennedy, E.P., Fed. Proc. 20:934 (1961).
18. Wise, E.M., Jr., and E. Elwyn, J. Biol. Chem. 240:1537 (1965).
19. Reiser, R., M.C. Williams and M.F. Sonels, J. Lipid Res. 1:241 (1960).
20. Bjornstad, P., and J. Bremer, Ibid. 7:38 (1966).
21. Gorin, E., and E. Shafrir, Biochem. Biophys. Acta 70:109 (1963).
22. Lands, W.E.M., Ann. Rev. Biochem. 34:313 (1965).
23. VanDeenen, L.L.M., and G.H. DeHaas, Ibid. 35:157 (1966).
24. Lands, W.E.M., M.L. Blank, L.J. Nutter and O.S. Privett, Lipids 1:224 (1966).
25. Reitz, R.C., W.E.M. Lands, W.W. Christie and R.T. Holman, J. Biol. Chem. 243:2241 (1968).
26. Nakamura, M., and O.S. Privett, Lipids 4:41 (1969).
27. Nakamura, M., and O.S. Privett, Ibid. 4:93 (1969).
28. Snyder, F., Anal. Biochem. 9:103 (1964).

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SHORT COMMUNICATIONS

α-Glycerolphosphate Dehydrogenase Activity and Levels of Glyceride-Glycerol Precursors in Mouse Mammary Tissues

ABSTRACT

Although α-glycerolphosphate dehydrogenase activity in slow growing mouse mammary tumors is only 5% of that in normal mammary glands of lactating mice, the cellular concentration of α-glycerolphosphate in both tissues is similar and is ca. 12 times that of dihydroxyacetone phosphate. Thus a positive correlation between the activity of glycerolphosphate dehydrogenase and the intracellular concentration of glyceride-glycerol precursors could not be demonstrated.

Glyceride synthesis occurs via a series of reactions subsequent to the acylation of either α-glycerolphosphate (GP) or dihydroxyacetone phosphate (DHAP) (1). However, in the case of the synthesis of glyceryl-ethers, only DHAP serves as the precursor (2,3). The proportion of glyceryl-ethers in lipid extracts from a variety of tumors is higher than that found in many tissues (4,5). Howard et al. (6) have pointed out a correlation between the low α-glycerolphosphate dehydrogenase (GPDH) activity and the high glyceryl-ether levels in neoplasms. It was suggested that a decrease in GPDH activity could result in increased levels of DHAP which in turn would be conducive for glyceryl-ether synthesis. Since GPDH is mainly responsible for the synthesis of GP, Hajra (7) has implied that the low level of enzyme activity reflects the insignificant role of the GP pathway for lipid synthesis in tumor cells. The present investigation was carried out to determine whether the intracellular levels of GP and DHAP are influenced by either the GPDH activity or the reductive environment of the tissue.

Tumor A, a spontaneous neoplasm found in an old virgin female C_3H mouse, was propagated by serial transplantation in our laboratory. Tumor C arose from a hyperplastic alveolar nodule outgrowth implanted into a cleared mammary fat pad of a 3-week-old C_3H mouse (8). This tumor has been serially transplanted subcutaneously into isologous females in the laboratory. Both tumor lines have been examined histologically and were found to resemble those primary tumors described previously (8-10). We have designated Tumor A as slow growing (8-10 weeks to attain 3-4 g in a 20 g mouse) and Tumor C as fast growing (3-4 weeks to attain 6-8 g in a 20 g mouse). All tumors used in this study were obtained from virgin mice when the tissue was ca. 3-6 g and only the firm non-necrotic tissue was used for analysis.

Lactating C_3H mice which actively suckled 5-8 pups for 17-19 days were used as the source of normal mammary tissue. Preparation of tissue homogenates, isolation of subcellular fractions and the assay of GPDH in the particle-free 100,000 x g supernatant fraction have been described previously (8-9). The estimation of GP, DHAP, lactate and pyruvate was carried out by standard procedures (11) with per-

TABLE I

α-Glycerolphosphate Dehydrogenase and Some Substrate Levels in Normal and Neoplastic Mouse Mammary Tissues[a]

Tissue	GPDH activity[b]	GP[c]	DHAP[c]	GP/DHAP	Lactate[c]	Pyruvate[c]	Lactate/pyruvate
Tumor A	20 ± 4	434 ± 14	37 ± 4	12	4632 ± 871	113 ± 19	41
Tumor C	10 ± 1	135 ± 24	68 ± 5	2	5299 ± 367	112 ± 14	47
Mammary gland	372 ± 34	518 ± 83	36 ± 1	14	6000 ± 955	117 ± 12	51

[a]The results are given as mean ± SD of four experiments with tissues taken from different animals.

[b]GPDH activity is expressed as nmoles of NADH oxidized/min/mg protein of the 6 x 10^6 g min supernatant fraction.

[c]The substrate concentrations are given as nmol/g wet wt tissue.

chloric acid extracts of tissues. The extracts were prepared within 1 min after killing the mice by cervical fracture. Protein concentration was determined by the method of Lowry et al. (12), using bovine serum albumin as standard.

As previously noted (8), the activity of GPDH in these mammary gland tumors was much lower than that found in the normal tissue counterpart (Table I). Although GPDH activity in Tumor A was only 5% of that in normal mammary glands of lactating mice, the cellular concentrations of DHAP in both tissues were similar. Furthermore the amount of GP in these tissues was 12-14 times that of DHAP. The value for the ratio GP/DHAP in Tumor C was much lower than in Tumor A.

It may be argued that the substrate ratio, GP/DHAP, may be altered due to a change in the NADH levels in the tissue rather than from the difference in the levels of GPDH. However, when we measured the cytoplasmic redox state of the cells by estimating the content of lactate and pyruvate (13), we found similar amounts of these two compounds in both normal and tumor tissues (Table I). Thus the cellular contents of GP and DHAP did not correlate with the levels of enzyme activity and appeared to be independent of coenzyme levels as well.

This lack of correlation between the levels of GPDH, GP and DHAP may be a general phenomenon and not confined only to mammary tumors. Other examples of a lack of correlation are as follows: The GPDH activity of Ehrlich ascites cells is 2.2 nmol/min/mg protein (14). Despite low dehydrogenase activity, the GP/DHAP ratio is rather high, namely 9.1 (15). In normal mouse liver, on the other hand, we have observed that the activity of GPDH in the particle free supernatant fraction was 290±43 nmol/min/mg protein and the content of GP and DHAP was 577±76 and 43±6.6 nmol/g wet wt, respectively. (It is possible that substrate concentrations could have changed during the time before homogenization in perchloric acid. However the values we obtained with liver using the same techniques as with mammary tissues were not significantly different from those reported by other workers [16-18] who used rapid freezing procedures.) In mouse liver, the value for the GP/DHAP ratio is therefore 13.4. In rat liver, Chance et al. (18) report a value of 11 for this ratio. Furthermore the GPDH activity of rat mammary gland varies from 0.56-3.49 nmol/min/g of fresh tissue during pregnancy and lactation, respectively (19). However, when we compared the reported GP and DHAP levels of these tissues (20), we again did not observe a positive correlation between substrate level and the dehydrogenase activity.

While DHAP content of a tissue is undoubtedly of importance for glyceride and particularly glyceryl-ether synthesis, the results of the studies presented and quoted here demonstrate that the process whereby this substrate level in the tissue is controlled may not reside in the GPDH activity alone.

Although glyceryl-ethers are present in a proportionately greater amount in the lipid extract of neoplasms than of normal tissues (4,5), glyceryl-ethers are not the major lipid constituent of neoplasms. In the case of the mouse mammary gland system, the normal tissue counterpart, lactating mammary gland contains more lipid (10%) than the mammary adenocarcinoma which has a lipid content of only 3% (10). Thus, in order for the tumor to possess more of these unusual lipids, the neoplasm must have in excess of three times as much of them on a proportional basis. The situation in the case of glands taken from virgin or pregnant mice is somewhat different, since the normal tissues in these instances contain even more lipid than the lactating gland. To our knowledge, analysis of the *absolute amounts* of ether lipids in mouse mammary tumors and their control tissues have not yet been carried out.

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REFERENCES

1. Lennarz, W.J., Ann. Rev. Biochem. 39:359 (1970).
2. Hajra, A.K., Biochem. Biophys. Res. Commun. 37:486 (1969).
3. Wykle, R.L., C. Piantadosi and F. Snyder, J. Biol. Chem. 247:2944 (1972).
4. Wood, R., and F. Snyder, J. Lipid Res. 8:494 (1967).
5. Snyder, F., and R. Wood, Cancer Res. 28:972 (1969).
6. Howard, B.V., H.P. Morris and J.M. Bailey, Ibid. 32:1533 (1972).
7. Hajra, A.K., JAOCS 49:Abstr. 64 (1972).

8. Kopelovich, L., S. Abraham, H. McGrath, K.B. DeOme and I.L. Chaikoff, Cancer Res. 26:1534 (1966).
9. Kopelovich, L., S. Abraham, H. McGrath and I.L. Chaikoff, Ibid. 27:800 (1967).
10. Bartley, J.C., H. McGrath and S. Abraham, Ibid. 31:527 (1971).
11. "Methods of Enzymatic Analysis," Edited by H.U. Bergmeyer, Academic Press, New York and London, 1965.
12. Lowry, O.H., M.J. Rosenbrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193:265 (1951).
13. Williamson, D.H., P. Lund and H.A. Krebs, Biochem. J. 103:514 (1967).
14. Agranoff, B.W., and A.K. Hajra, Proc. Nat. Acad. Sci. 68:411 (1971).
15. "Control Mechanisms in Respiration and Fermentation," Edited by B. Hess and B. Wright, Ronald Press, New York, 1963.
16. Zakim, D., R.S. Pardini, R.H. Herman and H.E. Sauberlich, Biochim. Biophys. Acta 144:242 (1967).
17. Shearer, T.R., and J.W. Suttie, J. Nutr. 100:749 (1970).
18. Chance, B., B. Schoener, K. Krejci, W. Russmann, W. Wesemann, H. Schnitger and Th. Bucher, Biochem. Z. 341:325 (1965).
19. Baldwin, R.L., and L.P. Milligan, J. Biol. Chem. 241:2058 (1966).
20. Baldwin, R.L., and W. Chang, J. Dairy Sci. 52:523 (1969).

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Glycerol Thioethers in Human Heart

ABSTRACT

Glycerol thioethers have been isolated from the nonpolar (neutral) lipids of several human hearts. The qualitative distribution of these thioethers was determined by gas liquid chromatography. The principal compounds found were the 16:0, 18:0 and 18:1 ether chains.

Glycerol thioethers have recently been isolated in our laboratory from bovine heart (1). We have also been using human heart tissue as the object of research dealing with the glycerol alkyl and alk-1-enyl ethers (2-5). Other workers have used human heart for similar studies involving glycerol alkyl and alk-1-enyl ethers (6-8). Thus it was felt that a study of the glycerol thioethers in human heart would be of interest. This paper reports these findings.

TABLE I

Qualitative Distribution of Glycerol Thioethers in Human Heart

Shorthand designation	H-6[a]	H-57	H-78
12:0	4.3	Trace[b]	2.2
13:0	1.2	3.7	Trace
14:0	9.8	8.6	7.8
15:0	1.7	2.3	1.9
Unknown	2.3	Trace	1.3
16:0	38.7	41.7	43.5
16:1	5.6	6.6	Trace
17:0	4.5	3.9	1.7
18:0	16.9	21.8	19.8
18:1	15.0	10.2	20.1

[a]Patient's age in years.
[b]Less than 1%.

The human hearts used in this study were obtained at autopsy and immediately frozen. These hearts were from patients whose case histories, combined with pathological evidence, showed them to be free of infarction.

Lipid extraction procedures, and the isolation and identification of glycerol thioethers have been described previously (1). One modification of the aforementioned procedure was the elimination of the necessity for making isopropylidene derivatives prior to separating the glycerol O and S ethers (9). These ethers can be separated by thin layer chromatography (TLC) on plates of Anasil G using a developing solvent of hexane-diethylether-acetic acid 30:70:1 v/v/v. In this solvent system the Rf's of the O and S ethers are 0.16 and 0.21, respectively.

Gas liquid chromatographic analyses of S-ether isopropylidene derivatives were carried out using a Packard dual column Model 7500 instrument equipped with a hydrogen flame detector. A 6 ft glass column of 14.5% ethylene glycol succinatemethyl silicone polymer (EGSS-X) coated on 100-120 mesh Gas-Chrome P was used isothermally at 185 C. Nitrogen was the carrier gas at a flow rate of 60 ml/min. Injector and detector temperatures were 290 C and 250 C, respectively. These conditons give quantitative results with isopropylidene derivatives of S-alkyl ethers of glycerol (9). Retention times were calculated relative to octadecyl glycerol thioether, and peak areas were determined as the product of peak height and width at half peak height. Percentages are given in

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9. Kopelovich, L., S. Abraham, H. McGrath and I.L. Chaikoff, Ibid. 27:800 (1967).
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terms of peak areas.

The results obtained from three human hearts are given in Table I. As can be seen in all hearts, the principal chain lengths present are the 16:0, 18:0 and 18:1 thioethers, and this is the same as that obtained for the corresponding alkyl and alk-1-enyl glycerol ethers of human heart, as well as various other tissues (10).

The presence of glycerol thioethers in human heart indicates that these lipids may be as widespread in occurence as their oxygen analogs (10). In normal human hearts the combined 1-O-alkyl and 1-alk-1-enyl ethers are present in amounts from ca. 11-27 μmol/100 mg lipid, depending on the patient's age (5). These values represent the total O-ethers obtained following reduction of total lipid extracts with vitride and isolation of the ether fractions by TLC (16). Alk-1-enyl ethers are assayed directly as hydrazones (12), and alkyl ethers are arrayed as hydrazones following oxidation with periodate (13). Approximately 75% of the total O-ethers are of the alk-1-enyl type. Thioethers, on the other hand, are present in much smaller amounts, and therefore quantitation is difficult. Using ca. 1 kg heart tissue and our hydrazone method (12), after periodate oxidation (13), the approximate value obtained for the isolated S-alkyl ethers was 0.09 μmol/100 mg lipid. We are presently investigating the possibility of S-alk-1-enyl ethers occurring naturally.

The biosynthetic origin of thioethers is yet to be determined. It is now established that alkyl glyceryl ethers originate enzymatically from a fatty alcohol and dihydroxyacetone phosphate, with the fatty alcohol contributing the oxygen to the ether bond (14). By analogy, we have shown in some preliminary work that glyceryl thioethers can be made by microsomes using dihydroxyacetone phosphate and a fatty mercaptan (15). An extensive investigation into this process is currently in progress.

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REFERENCES

1. Ferrell, W.J., and D.M. Radloff, Physiol. Chem. Phys. 2:551 (1970).
2. Radloff, J.F., and W.J. Ferrell, Ibid. 2:105 (1970).
3. Ferrell, W.J., D.M. Radloff and J.F. Radloff, Anal. Biochem. 37:227 (1970).
4. Ferrell, W.J., and J.F. Radloff, Mich. Cardiovascular Res. Forum 1:29 (1970).
5. Ferrell, W.J., and J.F. Radloff, Internatl. J. Biochem., 3:498 (1972).
6. Debuch, H., and M. Winterfeld, Z. Klin. Chem. 4:149 (1966).
7. Hughes, B.P., and F.F. Frais, Nature (London) 215:993 (1967).
8. Panganamala, R.V., L.A. Horrocks, J.C. Geer and D.C. Cornwell, Chem. Phys. Lipids 3:401 (1969).
9. Wood, R., C. Piantadosi and F. Snyder, J. Lipid Res. 10:370 (1969).
10. Horrocks, L.A., in "Ether Lipids: Chemistry and Biology," Edited by F. Snyder. Academic Press, New York, 1972, p. 226.
11. Snyder, F., M.L. Blank and R.L. Wykle, J. Biol. Chem. 246:3639 (1971).
12. Ferrell, W.J., J.F. Radloff and A.B. Jackiw, Lipids 4:278 (1969).
13. Gelman, R.A., and Gilbertson, J.R., Anal. Biochem. 31:463 (1969).
14. Snyder, F., W.T. Rainey, Jr., M.L. Blank and W.H. Christie, J. Biol. Chem. 245:5853 (1970).
15. Ferrell, W.J., and E. Desmyter, JAOCS, 49:83A (Abstr. 58) (1972).

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Developmental Changes in Lipid Composition of Chick Sciatic Nerve Myelin

ABSTRACT

Myelin isolated from the sciatic nerves of chicks by centrifugation on a discontinuous sucrose gradient was found to undergo marked changes in lipid composition during development from the 18 day embryonic to the adult stage, particularly in the phospholipid and cerebroside content. Significant changes in the content of individual phospholipids were also noted.

Myelin is formed in the central nervous system (CNS) by oligodendroglial cells. It has been reported that the lipid composition of

terms of peak areas.

The results obtained from three human hearts are given in Table I. As can be seen in all hearts, the principal chain lengths present are the 16:0, 18:0 and 18:1 thioethers, and this is the same as that obtained for the corresponding alkyl and alk-1-enyl glycerol ethers of human heart, as well as various other tissues (10).

The presence of glycerol thioethers in human heart indicates that these lipids may be as widespread in occurence as their oxygen analogs (10). In normal human hearts the combined 1-O-alkyl and 1-alk-1-enyl ethers are present in amounts from ca. 11-27 μmol/100 mg lipid, depending on the patient's age (5). These values represent the total O-ethers obtained following reduction of total lipid extracts with vitride and isolation of the ether fractions by TLC (16). Alk-1-enyl ethers are assayed directly as hydrazones (12), and alkyl ethers are arrayed as hydrazones following oxidation with periodate (13). Approximately 75% of the total O-ethers are of the alk-1-enyl type. Thioethers, on the other hand, are present in much smaller amounts, and therefore quantitation is difficult. Using ca. 1 kg heart tissue and our hydrazone method (12), after periodate oxidation (13), the approximate value obtained for the isolated S-alkyl ethers was 0.09 μmol/100 mg lipid. We are presently investigating the possibility of S-alk-1-enyl ethers occurring naturally.

The biosynthetic origin of thioethers is yet to be determined. It is now established that alkyl glyceryl ethers originate enzymatically from a fatty alcohol and dihydroxyacetone phosphate, with the fatty alcohol contributing the oxygen to the ether bond (14). By analogy, we have shown in some preliminary work that glyceryl thioethers can be made by microsomes using dihydroxyacetone phosphate and a fatty mercaptan (15). An extensive investigation into this process is currently in progress.

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REFERENCES

1. Ferrell, W.J., and D.M. Radloff, Physiol. Chem. Phys. 2:551 (1970).
2. Radloff, J.F., and W.J. Ferrell, Ibid. 2:105 (1970).
3. Ferrell, W.J., D.M. Radloff and J.F. Radloff, Anal. Biochem. 37:227 (1970).
4. Ferrell, W.J., and J.F. Radloff, Mich. Cardiovascular Res. Forum 1:29 (1970).
5. Ferrell, W.J., and J.F. Radloff, Internatl. J. Biochem., 3:498 (1972).
6. Debuch, H., and M. Winterfeld, Z. Klin. Chem. 4:149 (1966).
7. Hughes, B.P., and F.F. Frais, Nature (London) 215:993 (1967).
8. Panganamala, R.V., L.A. Horrocks, J.C. Geer and D.C. Cornwell, Chem. Phys. Lipids 3:401 (1969).
9. Wood, R., C. Piantadosi and F. Snyder, J. Lipid Res. 10:370 (1969).
10. Horrocks, L.A., in "Ether Lipids: Chemistry and Biology," Edited by F. Snyder. Academic Press, New York, 1972, p. 226.
11. Snyder, F., M.L. Blank and R.L. Wykle, J. Biol. Chem. 246:3639 (1971).
12. Ferrell, W.J., J.F. Radloff and A.B. Jackiw, Lipids 4:278 (1969).
13. Gelman, R.A., and Gilbertson, J.R., Anal. Biochem. 31:463 (1969).
14. Snyder, F., W.T. Rainey, Jr., M.L. Blank and W.H. Christie, J. Biol. Chem. 245:5853 (1970).
15. Ferrell, W.J., and E. Desmyter, JAOCS, 49:83A (Abstr. 58) (1972).

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Developmental Changes in Lipid Composition of Chick Sciatic Nerve Myelin

ABSTRACT

Myelin isolated from the sciatic nerves of chicks by centrifugation on a discontinuous sucrose gradient was found to undergo marked changes in lipid composition during development from the 18 day embryonic to the adult stage, particularly in the phospholipid and cerebroside content. Significant changes in the content of individual phospholipids were also noted.

Myelin is formed in the central nervous system (CNS) by oligodendroglial cells. It has been reported that the lipid composition of

TABLE I

Lipid Composition of Sciatic Nerve Myelin
of 18 Day Embryonic Chick, 1 Day, 4 Day, 7 Day Chick and Adult

Component	Age of chick					
	18 Day embryo	1 Day chick	4 Day chick	7 Day chick	Adult	Analysis
	Lipid content, % total lipid[a]					
Cholesterol	37.1 ± 1.93	34.8 ± 0.41	35.8 ± 1.41	33.7 ± 0.95	35.0 ± 0.90	$p>0.05$
Phospholipid	48.3 ± 2.42	46.0 ± 0.41	42.3 ± 0.42	41.5 ± 1.98	39.3 ± 1.06	$p<0.05$
Cerebroside	14.6 ± 0.54	19.2 ± 0.69	21.9 ± 1.57	24.7 ± 0.69	26.1 ± 0.31	$p<0.01$

[a]Values underscored by a common line are significantly different. The p values are determined from the difference in lipid content in the 18 day embryonic and adult myelin. The total lipid content of the sciatic nerve in 18 day embryos and adults was found to be 1.8 and 2.3 mg/mg protein, respectively, indicating an overall increase of these components during development.

CNS myelin changes markedly during development of many species. Horrocks (1) found decreased phospholipid content and increased cholesterol and cerebroside content of mouse brain myelin during development. Similar changes have been reported for rat brain (2) and rabbit brain myelin (3). It has been proposed that the CNS myelin is first deposited by the oligodendroglial cells as a primitive type of membrane similar to the oligodendroglial plasma membrane and is subsequently altered to a mature form (4).

Myelin is formed in the peripheral nervous system (PNS) by Schwann cells. The process is believed to be similar to that for the formation of CNS myelin (5); however developmental changes in the composition of PNS myelin have not been fully investigated. To undertake such an investigation myelin was isolated from the sciatic nerves of chicks of ages 18 day embryonic, 1 day, 4 day, 7 day chick and adult, and the lipid composition was determined.

The chicks were killed by decapitation, the sciatic nerves quickly removed and soaked in 0.05 M Tris 0.7 M glycine buffer pH 6.0 for 1 hr at 2 C. Myelin was isolated according to the procedure of Adams et al. (6) with some modifications. The nerves were homogenized in 0.32 M sucrose (6 vol) and centrifuged at 600 x g 20 min to remove nuclei and cellular debris. The supernatant was made ca. 0.8 M in sucrose by addition of an equal volume of 1.2 M sucrose and 4.8 ml layered on 5.4 ml of 1.2 M sucrose followed by 1.8 ml of 0.32 M sucrose layered on top. After centrifugation at 28,000 rpm for 90 min, in a Beckman SW 41 Ti rotor, the myelin band, which formed between the 0.32 M and 0.8 M sucrose, was removed, subjected to osmotic shock treatment according to Autilio et al. (7), and the centrifugation step repeated. The myelin, which banded in the same region of the gradient as previously, was removed and washed five times with water. Samples were removed for electron microscopy, and the remainder was freeze-dried and stored under nitrogen at -20 C until use. The purity of the myelin preparations was also checked by analysis of their content of nucleic acids (8)

TABLE II

Phospholipid Composition of Sciatic Nerve Myelin
of 18 Day Embryonic Chick, 1 Day, 4 Day, 7 Day Chick and Adult

Component	Age of chick					
	18 Day embryo	1 Day chick	4 Day chick	7 Day chick	Adult	Analysis
	Phospholipid content, % total phospholipid[a]					
Phosphatidyl serine	21.2 ± 0.87	17.8 ± 0.69	18.0 ± 0.69	17.0 ± 1.31	19.1 ± 0.46	$p>0.05$
Sphingomyelin	21.8 ± 1.65	22.3 ± 0.13	22.9 ± 0.87	24.3 ± 1.20	30.6 ± 0.98	$p=0.01$
Phosphatidyl choline choline	25.1 ± 1.20	21.7 ± 0.60	19.8 ± 0.51	18.4 ± 1.18	12.2 ± 0.25	$p<0.01$
Phosphatidyl ethanolamine	32.0 ± 2.35	38.3 ± 0.58	39.6 ± 0.46	40.4 ± 1.46	38.2 ± 0.31	$p=0.05$

[a]Values underscored by a common line are significantly different. The p values are determined from the difference in phospholipid content in the 18 day embryonic and adult myelin.

and activities of cytochrome oxidase (E.C. 1.9.3.1) (9) and lactate dehydrogenase (E.C. 1.1.1.27) (10). The average RNA and DNA content of the purified myelin preparations was a low 0.05% of the dry weight of myelin. There was no significant difference between the nucleic acid content of myelin from young or adult nerves. Furthermore the absence of cytochrome oxidase (E.C. 1.9.3.1) and lactate dehydrogenase (E.C. 1.1.1.27) activities in the purified myelin samples indicated negligible cytoplasmic contamination.

The dry myelin samples were weighed, dissolved in chloroform-methanol 2:1 v/v, and purified lipid extracts were prepared according to Folch et al. (11). Cholesterol (12), phospholipid (13) and cerebroside (14) contents of these purified lipid extracts were determined.

The results, expressed as per cent of total lipid, are shown in Table I. Values represent the mean ± SEM for three experiments. Myelin was extracted from sciatic nerves of 180 to 60 embryos or chicks and three hens for each experiment. Statistical analyses, based on the *t* test, were carried out to determine significant differences in lipid content with increasing age of the chick, and are included in Tables I and II. There was an increase in cerebroside content of sciatic nerve myelin with increasing age. The most significant increase occurred between the 18 day embryonic stage and the day-old chick. Also, a decreased phospholipid content was observed, most significantly between the 1 day and 4 day chick myelin. These results were comparable to those for CNS myelin (1-3). However no significant change occurred in the cholesterol content, at least in the age group studied.

To determine the content of individual phospholipids, aliquots of the purified lipid extract were analyzed by one dimensional thin layer chromatography using Absorbosil-4 (Applied Science Labs., Inc.) developed in chloroform-methanol-30% NH_3 14:6:1 v/v. The individual phospholipids were visualized by exposure to iodine vapor and identified by comparison of R_f values with authentic standards and also by the hydrolytic procedure of Dawson et al. (15). The spots were scraped off and eluted according to De Bohner et al. (16). The phospholipid content of each spot was determined and the results, expressed as per cent of total phospholipid, are shown in Table II. As indicated in the table, significant changes in the content of individual phospholipids occurred during development of chick sciatic nerve myelin. Phosphatidyl serine decreased only slightly with increasing age of the chick, while phosphatidyl ethanolamine showed a slight increase. Sphingomyelin and phosphatidyl choline showed the greatest changes, sphingomyelin increasing and phosphatidyl choline decreasing with increasing age of the chick. Similar changes have been reported for developing CNS myelin (1,3,17).

During the isolation procedure, a second band appeared between the 0.8 M and 1.2 M sucrose after centrifugation of the density gradient. The quantity of this fraction decreased with increasing age of the chick. Electron microscopy showed this fraction to be different from the myelin fraction. Whereas the myelin fractions of the different ages studied were found to contain only typical compact myelin, this second fraction contained mostly single membrane vesicles, but other unidentified fragments were present. A myelin-like fraction has been found by Agrawal et al. (18) and Banik and Davison (4) to be present in CNS. They proposed that this fraction is associated with myelin, in that it may be a stage in the formation of compact myelin. Preliminary investigation into the lipid content of this fraction has shown that it contains more phospholipid and less cholesterol and cerebroside than the purified myelin fraction. Experiments are in progress to further characterize this fraction. It may well be similar to that found in CNS.

In summary, purified myelin isolated from the sciatic nerve of chicks showed marked changes in lipid content during development. These changes in PNS myelin are comparable to those in CNS myelin. A second myelin-like fraction was isolated and may be associated with the myelination process.

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REFERENCES

1. Horrocks, L.A., J. Neurochem. 15:483 (1968).
2. Cuzner, M.L., and A.N. Davison, Biochem. J. 106:29 (1968).
3. Dalal, K.B., and E.R. Einstein, Brain Res. 16:441 (1969).
4. Banik, N.L., and A.N. Davison, Biochem. J. 115:1051 (1969).
5. Geren, B.B., Exp. Cell Res. 7:558 (1954).
6. Adams, C.W.M., Y.H. Abdulla, D.R. Turner and O.B. Bayliss, Nature (London) 220:171 (1968).

[1]These results represent a portion of a thesis, partial fulfillment of the requirements for the Ph.D. degree at Dalhousie University.

7. Autilio, L.A., W.T. Norton and R.D. Terry, J. Neurochem. 11:17 (1964).
8. Santen, R.J., and B.W. Agranoff, Biochim. Biophys. Acta 72:251 (1963).
9. Cooperstein, S.J., and A. Lazarow, J. Biol. Chem. 189:665 (1951).
10. Kornberg, A., in "Methods of Enzymology," Vol. I, Edited by S.P. Colowick and N.O. Kaplan, Academic Press, New York, 1955, p. 441.
11. Folch, J.P., M. Lees and G.H. Sloane-Stanley, J. Biol. Chem. 226:497 (1957).
12. Levine, J., and B. Zak, Clin. Chim. Acta 10:381 (1964).
13. Bartlett, G.R., J. Biol. Chem. 234:466 (1959).
14. Hess, H.H., and E. Lewin, J. Neurochem. 12:205 (1965).
15. Dawson, R.M.C., N. Hemington and J.B. Davenport, Biochem. J. 84:497 (1962).
16. De Bohner, S., E.F. Sato and T. De Cohan, J. Chromatr. 17:513 (1965).
17. Eng, L.F., and E.P. Noble, Lipids 3:157 (1968).
18. Agrawal, H.C., N.L. Banik, A.H. Bone, A.N. Davison, R.F. Mitchell and M. Spohn, Biochem. J. 120:635 (1970).

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Radiochemical Decomposition of Lactosyl-6-[^{3}H]-Ceramide

ABSTRACT

Galactosyl-6-[^{3}H]glucosyl ceramide was prepared by the sequential action of galactose oxidase and sodium borohydride-[^{3}H] reduction. A water-soluble radioactive contaminant appeared after a 2 month storage at -4 C. This was identified as lactose-[^{3}H] by both chromatographic and carrier dilution techniques.

Studies on the hydrolysis of the individual linkages present in the sphingolipids are severely limited, due to the relatively low activity of the enzymes catalyzing these reactions. Therefore conventional colorimetric or chromatographic procedures are usually either too insensitive or too tedious for routine assay of these hydrolases.

Radin and coworkers reported a two-step procedure for the introduction of tritium into the 6 position of the galactose moiety of both galactosyl (1) and lactosyl (2) ceramides. The first step involves treatment of the sphingolipid with the commercially available galactose oxidase to yield the corresponding 6-aldehydo galactoside which is then partially purified and subjected to reduction with sodium borohydride-[^{3}H]. Galactosyl-6[^{3}H]-ceramide prepared by this procedure has been employed to document the enzymatic defect in Krabbes disease (3) and galactosyl-6-[^{3}H]-glucosylceramide to detect the ceramide lactosidoses defect (4). Galactose oxidase has been reported to

FIG. 1. Thin layer chromatography and radioactive scans of stored lactosyl-[^{3}H] ceramide before and after Folch partitioning. Lane A, standard ceramide; lane B, standard mixture; lane C, starting lactosyl-[^{3}H] ceramide; lane D, lactose standard; lane E, standard mixture; lane F, lactosyl-[^{3}H] ceramide after partitioning; lane G, galactose standard; lane H, lactosyl ceramide standard. Chloroform-methanol-water 65:35:8 v/v was the solvent employed.

7. Autilio, L.A., W.T. Norton and R.D. Terry, J. Neurochem. 11:17 (1964).
8. Santen, R.J., and B.W. Agranoff, Biochim. Biophys. Acta 72:251 (1963).
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10. Kornberg, A., in "Methods of Enzymology," Vol. I, Edited by S.P. Colowick and N.O. Kaplan, Academic Press, New York, 1955, p. 441.
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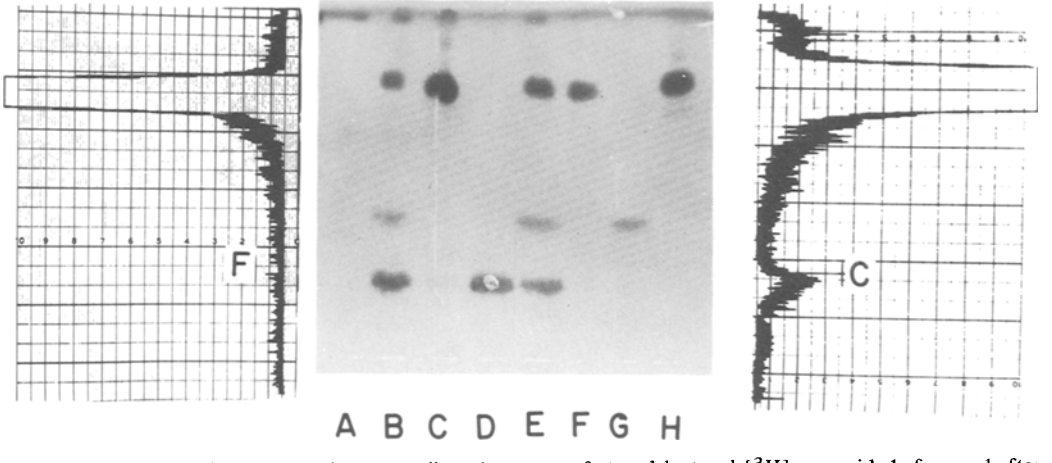

FIG. 1. Thin layer chromatography and radioactive scans of stored lactosyl-[^{3}H] ceramide before and after Folch partitioning. Lane A, standard ceramide; lane B, standard mixture; lane C, starting lactosyl-[^{3}H] ceramide; lane D, lactose standard; lane E, standard mixture; lane F, lactosyl-[^{3}H] ceramide after partitioning; lane G, galactose standard; lane H, lactosyl ceramide standard. Chloroform-methanol-water 65:35:8 v/v was the solvent employed.

oxidize the galactose in several of sphingoglycolipids (5), and recently the synthesis of a variety of radioactive sphingolipids based upon this procedure appeared (6).

This report documents the formation of lactose-[^{3}H] as the water-soluble material during the storage of galactosyl-6-[^{3}H]-glucosyl ceramide.

Mixed brain gangliosides were prepared as previously described (7) and subjected to acid hydrolysis; lactosyl ceramide was recovered from the hydrolyzate mixture and subjected to oxidation with galactose oxidase and reduction with sodium borohydride-[^{3}H] according to the method of Radin et al. (2). The final product showed only a single radioactive material cochromatographing with lactosyl ceramide standards with Silica Gel G thin layer plates in two different solvent systems. The sample having a final specific activity of 500 dpm/nmol was stored at -4 C, dissolved in chloroform-methanol 2:1. Phenol-H_2SO_4 was employed for carbohydrate determination (8). Silica Gel G plates for thin layer chromatography were obtained from Analtech (Wilmington, Del.). Spots were visualized by spraying with 5% sulfuric acid in methanol spray and heating.

TABLE I

Carrier Dilution of Radioactive Water-soluble -[^{3}H] Material Formed from Lactosyl Ceramide and of Galactose-^{14}C with K^+ Lactonate

	cpm/mg	
Crystallization	Water-soluble -[^{3}H]	Galactose-^{14}C
First	43.7	137.8
First	44.1	17.2
Second	45.1	2.0

When this material was employed as a substrate for β-galactosidase after being stored for 2 months, it was observed that the "blank" values of incubation had increased considerably, suggesting a nonenzymic breakdown of this substrate. Thin layer chromotographic separation and scanning radioactivity revealed the

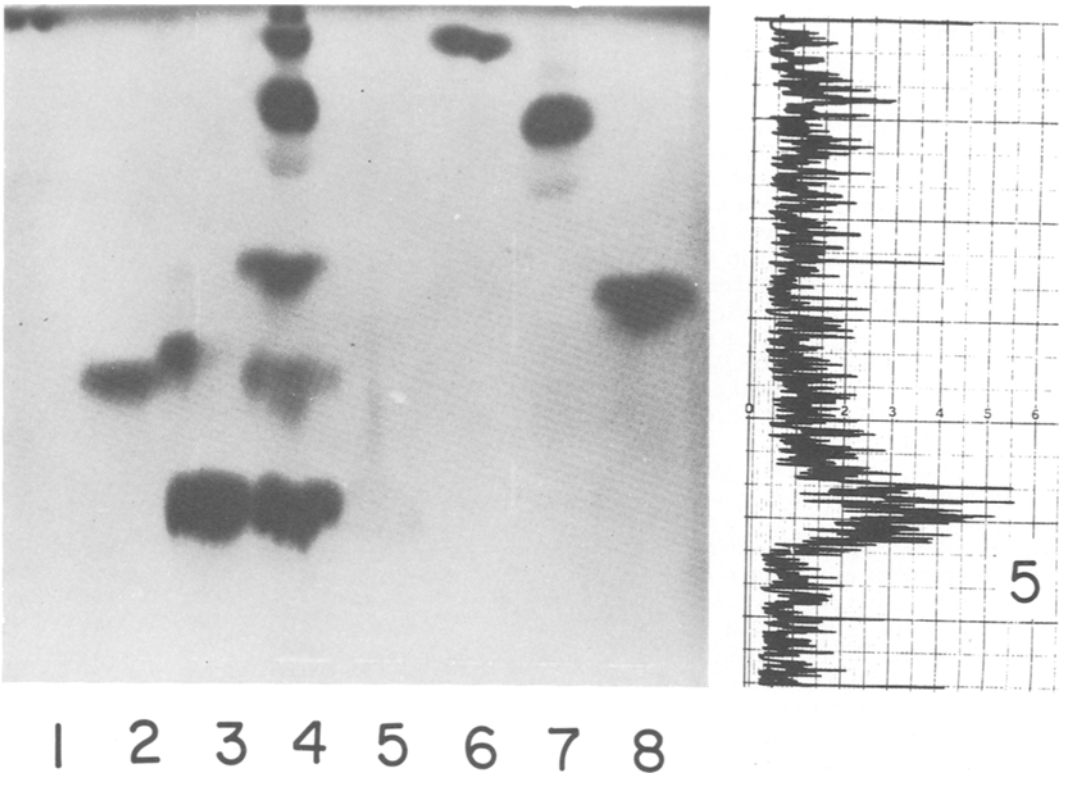

FIG. 2. Thin layer chromatography and radioactive scan of water-soluble material from lactosyl-[^{3}H] ceramide. Lane 1, ceramide standard; lane 2, galactose standard; lane 3, lactose standard; lane 4, mixture of standards; lane 5, water-soluble upper-phase material; lane 6, galactose cerebroside standard; lane 7, lactosyl ceramide standard; lane 8, methyl-β-galactoside standard. Chloroform-methanol-water 65:35:8 v/v was the solvent employed.

presence of two radioactive materials (Fig. 1, lane C). In addition to the lactosyl ceramide, a material migrating with lactose was observed. The sample was subject to the Folch et al. partitioning procedure (9) and the lower organic phase subject to chromatographic examination (Fig. 1, lane F). It is obvious that these extractions had removed a water-soluble contaminant, and this amounted to ca. 1.6% of the total radioactivity in the sample.

The upper phase from the partitioning was concentrated to dryness, dissolved in a small amount of water and passed through a mixed bed of Dowex 50-H^+ and Dowex-1-Acetate. The effluent was lyophilized, dissolved in a small quantity of water and an aliquot subjected to thin layer chromatography. A single radioactive material, outlined in Figure 2 cochromatographing with lactose, was observed (Fig. 2, lane 5). This solvent system adequately separated lactose, galactose, methyl galactoside and lactosyl ceramide. Further evidence for the material being lactose was obtained by classical carrier dilution techniques. An aliquot containing 5500 dpm was mixed with 100 mg nonradioactive lactose and subjected to hypoiodite oxidation (10). The insoluble potassium lactonate salt formed was harvested, weighed and recrystallized from a mixture of water, methanol and isopropylether; these results are presented in Table I. It is obvious that constant specific activity was readily attained; the value of 45 cpm/mg is in close agreement to the theoretical value of 55 cpm/mg. A control experiment was carried out in which 100,000 cpm galactose-1-^{14}C was mixed with 100 mg lactose, and the mixture subjected to this oxidation and recrystallization procedure. The final specific activity of the lactonate in this case was 2 cpm/mg, which indicates that 99.8% of the galactose was removed from the starting mixture.

The formation of lactose rather than galactose or methyl galactoside from lactosyl ceramide is unexpected. Hydrolytic studies were originally carried out to determine the carbohydrate sequence of gangliosides. Except for one poorly documented report of lactose liberation (11), most authors have been able to detect only galactosamine-containing disacchardies (12) from such hydrolyzates. This has been ascribed to the greater resistance of the sphingosine-glucose bond to hydrolysis than the other glycosidic linkages present in gangliosides.

This laboratory has reported the appearance of phosphorylcholine-^{14}C (13) and choline-^{14}C (14) after storage of synthetic sphingomyelin-^{14}C samples. In a similar manner, glucose-^{14}C have been detected in solutions of glucosyl-^{14}C ceramide (15) and galactosyl-^{14}C ceramides (1), prepared in this laboratory after storage. The finding of lactose-[^{3}H] in the lactosyl ceramide preparation further documents the potential difficulties in using such compounds for in vitro studies. It is therefore necessary to extract the lipid solution according to the Folch procedure and to check for impurity on thin layer chromatography prior to use as a substrate in experiments.

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REFERENCES

1. Hajra, A.K., D.M. Bowen, Y. Kishimoto and N.S. Radin, J. Lipid Res. 7:379 (1966).
2. Radin, N.S., L. Hof, R. Bradley and R.O. Brady, Brain Res. 14:497 (1969).
3. Suzuki, K., and Y. Suzuki, Proc. Nat. Acad. Sci. (U.S.) 66:302 (1970).
4. Dawson, G., and A. Stein, Science 170:557 (1970).
5. Bradley, R.M., and J.N. Kanfer, Biochim. Biophys. Acta 84:210 (1964).
6. Suzuki, K., and Y. Suzuki, J. Lipid Res. 13:687 (1972).
7. Kanfer, J.N., in "Methods in Enzymology," Vol. 14, Edited by S.P. Colowick and N.O. Kaplan, Academic Press, 1969, p. 660.
8. Dubois, M., K.A. Gillis, J.K. Hamilton, P.A. Reebers and F. Smith, Anal. Chem. 28:350 (1956).
9. Folch, J., M. Lees and G.H. Sloane-Stanley, J. Biol. Chem. 226:497 (1957).
10. Moore, S., and K.P. Link, Ibid. 133:293 (1940).
11. Kuhn, R., and H. Wiegandt, Chem. Ber. 96:866 (1963).
12. Svennerholm, L., J. Lipid Res. 5:145 (1964).
13. Kanfer, J.N., and M. Williams, Chem. Phys. Lipids 6:121 (1971).
14. Petrovich, G., R.A. Mumford and J.N. Kanfer, Ibid. in press.
15. Kanfer, J.N., J. Biol. Chem. 240:609 (1965).

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Fatty Acid Composition of Rat Hearts as Influenced by Age and Dietary Fatty Acids[1]

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ABSTRACT

Fatty acid composition of neutral and polar lipid fractions from rat hearts was determined in rats of different ages as their diet source changed. Piebald rats were weaned at 21 days and were fed standard lab chow. Lipids from rat hearts, mothers milk and lab chow were purified on a Sephadex G-25 fine column and separated into neutral and polar lipid fractions by silicic acid column chromatography. These lipid fractions were then hydrolyzed and methylated with BF_3 in methanol, prior to gas liquid chromatographic separation on a 1/8 in. x 10 ft aluminum column of 15% EGS on 80-100 mesh acid-washed Chromosorb W. Three major fatty acids in the neutral lipid fraction comprised 72% of total neutral lipid fatty acids from young hearts. At sexual maturity (at least 74 days old) $C_{18:1}$ was the major fatty acid, followed by $C_{16:0}$ and $C_{18:0}$. The same three fatty acids comprised 83% of total polar lipid fatty acids, but $C_{18:0}$ was the major fatty acid, followed by $C_{16:0}$ and $C_{18:1}$. The fatty acid composition of dietary lipids influenced the total neutral lipid fatty acid composition of the rat heart, but had little influence on the fatty acid composition of the polar lipid fraction.

INTRODUCTION

It is a known concept that muscles utilize mainly carbohydrates for energy purposes and, since the heart is a muscle, the same concept has been proposed. However research by Bing et al. (1) has revealed that the heart can and does derive energy from fatty acid metabolism. Studies have also been done to show the importance of fatty acids in the rat heart in vitro (2,3), perfused rat hearts (4-6) and rat heart cells in culture (7). In the latter system, the importance of linoleic and palmitic acids has been explored.

In maintaining rat heart cells in culture, it was observed in our laboratory that the heart tissue must be from an animal that is under 1 week of age, or beating does not occur. It was thought that availability of certain fatty acids in the young immature rat heart may be a prerequisite for the beating phenomenon. This idea prompted the following research.

Work has been performed by Glende and Cornatzer (3) on the fatty acid composition of phospholipids in the rat heart, but from animals beyond the age of sexual maturity. Stein and Stein (8) also examined palmitic and linoleic acids metabolism in hearts obtained from fatty acid-deficient rats. However these animals were beyond the age of weaning and very near the age of sexual maturity. Therefore it was the purpose of this work to determine what changes, if any, occur in rat heart fatty acids in neutral and polar lipid fractions as animals aged and diet changed from rat milk to laboratory chow.

METHODS

Sample Preparation

Hearts from anesthetized Piebald rats were removed by severing blood vessels and connective tissues just above the atria. The hearts were washed in isotonic saline, blotted dry with filter paper and wrapped in heavy duty aluminum foil. Next, they were immersed in liquid nitrogen, placed on a masonite board, and struck with a hammer. The crushed hearts were placed in a 10 ml centrifuge tube containing 5 ml absolute methanol and stored under nitrogen at -35 C.

The diet used in this study was a standard Ralston Purina laboratory chow designed for rats. The same diet was used through the testing period. Therefore any changes that occurred were relative to the fatty acids available in the lab chow.

Extraction of Total Lipids

Lipids were extracted by filtering the crushed hearts and methanol through Whatman no. 1 filter paper and washing the residue with 20 volumes chloroform-methanol 2:1, usually 20-40 ml. The residue was then extracted with 20 ml chloroform. The combined filtrates were evaporated under a stream of nitrogen or on a

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TABLE I

Fatty Acid[a] Composition of Neutral Lipid Fraction from Rat Hearts as Related to Dietary Fatty Acids

	Total fatty acids,[b] %						
Age, days	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:4}$
Rat milk[c]	Trace[d]	23.2 ± 2.1	3.6 ± 0.4	4.7 ± 0.3	42.0 ± 0.1	21.6 ± 0.1	5.8 ± 1.2
1	2.7 ± 0.4	25.0 ± 2.0	5.5 ± 0.4	17.9 ± 0.4	28.7 ± 1.6	10.8 ± 1.8	9.5 ± 1.4
3	2.7 ± 0.6	32.7 ± 2.2	5.5 ± 0.6	15.9 ± 0.4	29.4 ± 1.0	6.8 ± 1.4	7.0 ± 1.6
6	Trace	26.0 ± 1.8	3.5 ± 0.5	17.5 ± 0.9	30.7 ± 1.3	8.7 ± 0.8	13.1 ± 2.4
20	1.5 ± 0.2	23.4 ± 1.6	1.4 ± 0.5	13.7 ± 2.5	33.7 ± 1.6	16.1 ± 1.2	10.2 ± 1.5
42	1.6 ± 0.3	18.8 ± 0.6	2.4 ± 0.2	17.5 ± 1.1	30.6 ± 0.4	23.2 ± 1.5	6.0 ± 0.4
74	1.0 ± 0.2	17.8 ± 0.4	2.6 ± 0.3	17.1 ± 0.9	32.2 ± 0.5	26.1 ± 0.4	3.1 ± 0.6
Rat chow[e]	Trace	15.1 ± 0.6	2.3 ± 0.1	8.3 ± 0.1	35.4 ± 0.2	37.3 ± 0.7	Trace

[a]Measured as methyl ester.
[b]Values reported as average percentage of total fatty acids ± SE.
[c]Approximately 99% neutral lipid.
[d]Less than 1%.
[e]Approximately 95% neutral lipid.

Buchler Evapomixer at 30 C. The solvent-free crude extract was stored under nitrogen until used.

Removal of Nonlipid Contaminants from Crude Lipid Extracts

Lipids were purified by the method of Wells and Dittmer (9), whereby the lipids were dissolved in chloroform-methanol-water 60:30:4.5 and placed on a 10 ml microburet chromatography column equipped with a teflon stopcock and containing 1 g Sephadex G-25 fine. Lipids were added to the column in 5 ml quantities of the solvent mixture, but never more than 15 ml was added. The eluent flow rate was maintained at 0.1 ml/min. The column was next eluted with 5 ml chloroform-methanol 2:1 at the same flow rate. The solvents were combined and evaporated under a stream of nitrogen.

Separation of Purified Lipids into Neutral and Polar Lipid Fractions

Lipids freed of nonlipid contaminants were separated into neutral and polar lipids according to the method of Börgstrom (10). Depending on lipid concentrations, 3-5 g activated silicic acid (Unicil 100-200 mesh) was mixed with an equal amount of HyFlow Supelcel (Celite) and placed in a 100 ml buret equipped with a Teflon stopcock. The silicic acid was previously activated by heat at 100 C for 30 min. One gram activated silicic acid would bind ca. 30 mg polar lipids. The packed chromatography columns were washed with 50 ml chloroform and charged with 5 ml of a lipid solution in chloroform.

Neutral lipids were eluted with 50-70 ml chloroform and polar lipids were eluted with 75-100 ml absolute methanol. The solvent from the lipid fractions was removed by evaporation under a stream of nitrogen while heating the lipid solution with an IR lamp. Neutral and polar lipids were stored under nitrogen at -70 C. Stability studies showed little or no change in fatty acid composition under these conditions.

Preparation of Methyl Esters from Lipid Fractions

Fatty acid methyl esters were prepared according to the method of Morrison and Smith (11). A weighed lipid sample was placed in a 4 ml screw cap vial containing 2 ml benzene. One milliliter 12% BF_3 in methanol was added to the vial. The vial was sealed with a Teflon-lined cap and placed in a water bath at 100 C for 45 min after which the vial was removed, cooled, and 1 ml water added to stop the reaction. The mixture was shaken vigorously for 1 min and the phases allowed to separate. The benzene layer was placed in a 3 ml centrifuge tube and the solvent evaporated under a stream of nitrogen. Methyl esters were never prepared or stored more than 6 hr before chromatography. Stability studies showed that little or no change occurred in fatty acids under these conditions.

On testing for oxidative losses during isolation procedures, the stability of tetraenoic, pentaenoic and hexaenoic was not specifically examined.

Gas Liquid Chromatography

Chromatography was performed on an Aerograph gas chromatograph model 204B equipped with a dual flame ionization detector, which was standardized according to the special report of Horning et al. (12). Methyl esters were identified and quantitated on a 1/8 in. x 10 ft

TABLE II

Fatty Acid[a] Composition of Polar Lipid Fraction from Rat Hearts as Related to Dietary Fatty Acids

Age, days	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:4}$
Rat milk[c]	Trace[d]	23.3 ± 2.1	3.6 ± 0.4	4.7 ± 0.3	42.0 ± 0.1	21.6 ± 0.1	5.8 ± 1.2
1	Trace	14.4 ± 2.9	1.7 ± 0.4	33.1 ± 1.8	24.4 ± 1.0	11.4 ± 1.2	14.6 ± 2.4
3	Trace	23.6 ± 1.8	2.1 ± 0.6	38.3 ± 1.3	23.9 ± 1.4	9.7 ± 3.0	1.9 ± 1.3
6	Trace	27.8 ± 0.5	1.7 ± 0.3	45.2 ± 1.2	19.5 ± 0.4	3.8 ± 0.7	1.0 ± 0.5
20	Trace	22.2 ± 0.6	Trace	54.8 ± 1.2	14.4 ± 0.3	6.6 ± - .7	1.4 ± 0.6
42	Trace	16.7 ± 2.1	0.9 ± 0.1	49.2 ± 1.4	11.5 ± 0.7	12.9 ± 3.7	8.5 ± 2.4
74	Trace	11.8 ± 0.7	0.8 ± 0.2	40.4 ± 2.1	13.4 ± 0.8	24.0 ± 2.0	9.4 ± 2.0
Rat chow[e]	Trace	15.1 ± 0.6	2.3 ± 0.1	8.3 ± 0.1	35.4 ± 0.2	37.3 ± 0.7	Trace

[a]Measured as methyl ester.

[b]Values reported as average percentage of total fatty acids ± SE.

[c]Approximately 99% neutral lipid.

[d]Less than 1%.

[e]Approximately 95% neutral lipid.

aluminum column of 15% EGS on 80-100 mesh, acid-washed Chromosorb W. The second column used for identification of methyl esters was a 1/8 in. x 5 ft stainless steel column of 5% SE-30 on 80-100 Chromosorb P. The methyl esters were also identified by cochromatography with methyl ester standards from Supelco Inc.

Throughout separation of the methyl esters column temperature was held at 125 C for 5 min after injection, then heated to 185 C and maintained at this temperature the remainder of the run. The peaks were recorded on a Speedomax W recorder equipped with a Disc integrator model 224. Fatty acid esters were quantitated by converting the disc units on a standard curve for the range and attenuation of the electrometer. Each set of values represents the fatty acid compositon of two to three rat hearts with each gas chromatographic analysis done in duplicate.

RESULTS AND DISCUSSION

Data in Table I indicate there are three major fatty acids in the rat heart comprising ca. 72% of the total neutral lipid fatty acids found there. Lipids from 1-day-old rat hearts had the $C_{18:1}$ fatty acid as the major fatty acid, followed by $C_{16:0}$ and $C_{18:0}$. Lipids extracted from the hearts of sexually mature rats (74 days old) had $C_{18:1}$ and $C_{18:2}$ fatty acids, comprising 58% of the total fatty acids and $C_{16:0}$ comprising 35%. The $C_{14:0}$, $C_{16:1}$ and $C_{20:4}$ fatty acids' relative concentrations decreased slightly with age, while those of $C_{18:0}$ were essentially unchanged. These data indicate that percentages of saturated fatty acids in the rat hearts decreased with age, while percentage of unsaturated fatty acids increased.

In the procedures outlined in this study the only polyenoic fatty acids detected in significant amounts were $C_{18:2}$, $C_{18:3}$ and $C_{20:4}$. If other polyenoic fatty acids were present they should have been detected by the gas liquid chromatography procedures employed with the exception of C_{24} fatty acids and larger.

Table II presents data on the fatty acid composition of the polar lipids from rat hearts as a function of age. Polar lipids from 1-day-old rats had $C_{18:0}$ as the major fatty acids, followed by $C_{18:1}$. Upon sexual maturation of the rat, $C_{18:0}$ was still the major fatty acid in the polar lipid, but it was followed by $C_{18:2}$, $C_{18:1}$ and $C_{16:0}$. In the young heart the monoenes and dienes composed 37% of total fatty acids of the polar lipid fraction. In the mature heart saturated fatty acids comprised 52% of the total polar lipid fraction. This is a reversal of the fatty acid composition from the neutral lipids at the same time intervals. The $C_{16:0}$ and $C_{18:0}$ percentages of the polar lipid fraction increase from day 1 until weaning, after which their percentage compositions decreased. $C_{18:1}$ decreases steadily from day 1 until sexual maturity. $C_{18:2}$ composition decreases until weaning and then continues to increase. $C_{20:4}$ composition decreases abruptly after birth, from day 1 until day 3, and then begins to increase in relative percentages near sexual maturity. The trend for changes in saturated fatty acid composition of the polar lipid fraction shows a steady increase up to ca. 3 weeks and then a decrease. Unsaturated fatty acids show a reverse trend over the same time period. At day 20 the $C_{18:0}$ and $C_{16:0}$ fatty acid comprise 77% of fatty acids in the polar lipids and decrease to 52% at sexual maturity. Aging and sexual maturity in the rat is associated with an increase in unsaturated fatty acids

TABLE III

Fatty Acid[a] Composition of Rat Milk and Rat Lab Chow

Fatty acid	Total fatty acids,[b] %	
	Rat milk[c]	Rat lab chow[d]
$C_{14:0}$	Trace[e]	Trace
$C_{16:0}$	23.3 ± 2.1	15.1 ± 0.6
$C_{16:1}$	3.6 ± 0.4	2.3 ± 0.1
$C_{18:0}$	4.7 ± 0.3	8.3 ± 0.1
$C_{18:1}$	42.0 ± 0.1	35.4 ± 0.2
$C_{18:2}$	21.6 ± 0.1	37.3 ± 0.7
$C_{18:3}$	Trace	Trace
$C_{20:4}$	5.0 ± 1.2	Trace

[a]Measured as methyl ester.

[b]Values reported as average percentage of total fatty acids ± SE.

[c]Approximately 99% neutral lipid.

[d]Approximately 95% neutral lipid.

[e]Less than 1%.

of the polar lipid fraction of the rat heart. In the perfused rat heart Stein and Stein (8) found that in the presence of equal molar amounts of palmitic and linoleic acid in the perfusate more linoleic acid was incorporated into phospholipids and more palmitic acid into neutral lipids. In the whole rat heart where a "normal" dietary fatty acid composition is available, this type of pattern was not observed.

The per cent fatty acid composition of the rat milk and rat laboratory chow were next compared to the per cent neutral and polar lipid fatty acids from rat hearts at various ages of development. The data in Table III indicate that rat chow contains slightly more saturated fatty acids than rat milk lipids. Approximately 63% of the fatty acids in rat milk is $C_{18:1}$ and $C_{18:2}$, and ca. 73% of the fatty acids in rat chow is composed of these two acids. The amount of $C_{16:0}$ is higher in rat milk than in rat chow, and ca. 6% of the fatty acids is $C_{20:4}$. The fatty acid compositions of the neutral lipid fraction from rats hearts are shown at days 1, 3, 6, 20, 42 and 74. The day 1 data would indicate the composition of the hearts, supplied by exogenous lipids from the mother's blood. Data at day 20 should represent changes from growth using rat milk as an exogenous source of lipids. Days 42 and 74 were chosen to represent an intermediate time between weaning and sexual maturity, respectively. The relative percentages of various fatty acids in raw chow are shown in the last line. Changes in composition between rat milk and 1-day-old rat hearts are shown mainly in $C_{18:0}$, $C_{18:1}$ and $C_{18:2}$. Less $C_{18:0}$ but more $C_{18:1}$ and $C_{18:2}$ are found in rat milk compared to the rat heart. By day 20 the data indicate a trend toward a fatty acid composition approaching the rat milk composition. The only exception is in the $C_{20:4}$ composition. Its relative concentration continues to increase with age. The relative percentages of fatty acids are quite similar for 42-day-old animals and 74-day-old rats. The major differences in composition between rat chow and the rat heart at day 74 are seen in the $C_{18:0}$, $C_{18:2}$ and $C_{20:4}$. The composition of $C_{18:0}$ in the rat chow is less than that in the heart, and $C_{18:2}$ is greater in the rat chow than in the heart. The lower level of $C_{20:4}$ in the sexually mature rat's heart may reflect its absence in rat chow. It thus appears that rat heart neutral lipid fatty acids are determined largely by diet, but that rat heart tissues still have some control of the overall composition. This indicates either selective retention or useage of particular fatty acids by the rat heart neutral lipid fraction.

The data in Table II compare the relative fatty acid composition of the polar lipid fraction from rat hearts of various ages and the fatty acid composition of rat milk and rat chow. Comparisons are made in a manner similar to that for the neutral lipid fraction discussed in the previous paragraph. These data indicate a rather specific fatty acid composition for polar lipid fatty acids and one quite independent of fatty acids in the dietary source. $C_{18:0}$ fatty acid is present in rat milk and rat chow in low concentration, but it comprises over 50% of fatty acids in the 20-day-old rat heart. $C_{18:1}$ fatty acid is present in high concentration in rat milk and rat chow, but comprises 11-14% of the fatty acids in rat heart. $C_{16:0}$ and $C_{16:1}$ do reflect their concentrations in the milk and chow. $C_{20:4}$ is ca. 15% of fatty acids in the 1-day-old rat heart, but decreases in concentration abruptly at time of weaning and increases thereafter to ca. 10% at sexual maturity. These data support the results of Dobiasova and Kodalvsky (13) and Baldwin and Cornatzer (14), who found that fatty acid composition of triglycerides and phospholipids of some organs of the rat, during postnatal development, showed marked changes in fatty acid composition of the different organs, but these changes could not always be related to the changes in the composition in the diet.

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REFERENCES

1. Bing, R.J., A. Siegel, I. Ungar and M. Gilbert,

Amer. J. Med. 16:504 (1954).
2. Rothlin, M.E., and R.J. Bing, J. Clin. Invest. 40:1380 (1961).
3. Glende, E.A., Jr., and W.E. Cornatzer, Biochim. Biophys. Acta 125:310 (1966).
4. Olson, R.E., and R.J. Hoeschen, Biochem. J. 103:796 (1967).
5. Evans, J.R., L.H. Opie and J.C. Shipp, Amer. J. Physiol. 205:766 (1963).
6. Willebrands, A.F., Biochim. Biophys. Acta 84:607 (1964).
7. Gerschenson, L.E., I. Harary and J.F. Mead, Ibid. 131:50 (1967).
8. Stein, O., and Y. Stein, Ibid. 84:621 (1964).
9. Wells, M.A., and J.C. Dittmer, Biochemistry 2:1259 (1963).
10. Borgstrom, B., Acta Phys. Scandinav. 25:101 (1952).
11. Morrison, W.R., and L.M. Smith, J. Lipid Res. 5:600 (1964).
12. Horning, E.C., E.H. Ahrens, Jr., S.R. Lipsky, F.H. Mattson, J.F. Mead, D.A. Turner and W.H. Goldwater, Ibid. 5:20 (1964).
13. Dobiasova, M., P. Hahn and O. Kaldovsky, Biochim. Biophys. Acta, 84:538 (1964).
14. Baldwin, J., and W.E. Cornatzer, Lipids 3:362 (1968).

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Polyprenols of Beef and Human Pituitary Glands

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ABSTRACT

An unknown lipid previously isolated in our laboratory from beef and human pituitaries was shown to have the same structure as dolichol from pig liver. Beef pituitaries contained ca. 130 mg/kg wet tissue, of which ca. 25% was esterified to fatty acids. Human pituitaries contained ca. 1.4 g/kg, mainly as the free alcohol.

INTRODUCTION

An investigation of the lipid composition of beef and human pituitaries disclosed an unknown lipid in the triglyceride fraction, and preliminary investigation indicated that it was an unsaturated alcohol (1). The present paper provides evidence that it is a polyprenol having the same structure as dolichol from pig liver.

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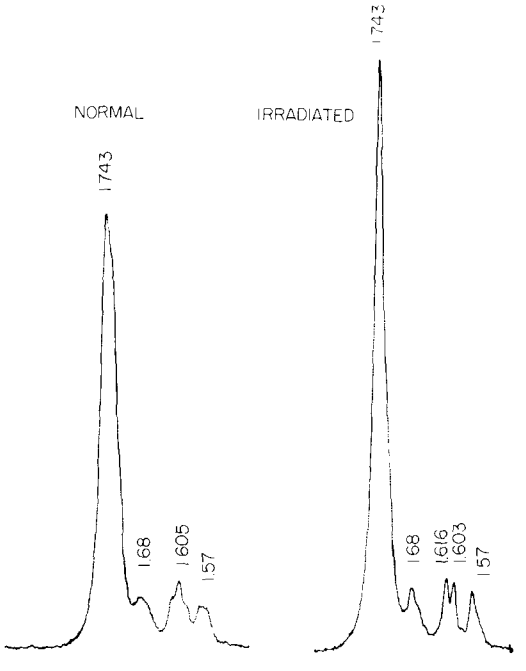


FIG. 1. Double bond methyl region (1.5-1.8 ppm) of NMR spectrum of beef pituitary polyprenols in C_6D_6 expanded at 250 Hz sweep width; normal and with double bond protons irradiated at 5.26 ppm.

MATERIALS AND METHODS

Beef pituitaries, freed of adipose and connective tissues, were obtained from F. Manion, Research Lab., Canada Packers Ltd., Toronto, Ontario, and human pituitaries from D.M. Mills, St. Joseph's Hospital, London, Ontario. Pituitaries were stored frozen until extracted. All solvents and other chemicals were either Reagent Grade or conformed to ACS specifications. Pyridine was dried over KOH and distilled before use.

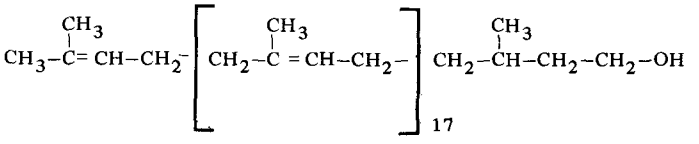
The lipids were extracted and separated essentially as described previously (1), but for isolation of larger quantities of the unknown lipid from beef pituitaries the method was scaled up as follows: Whole beef pituitaries were pooled in 50 g lots, homogenized with a Virtis homogenizer and extracted with 1000 ml $CHCl_3/CH_3OH$ 2:1 v/v overnight at room temperature. The mixture was filtered, the filtrate washed with 200 ml water, evaporated to dryness, and stored as a $CHCl_3$ solution at 4 C.

The crude lipid extracts were chromatographed on acid-treated Florisil (30 g, 2.4 x 30 cm, column load 6-7 g). Nonpolar lipids were eluted with 300 ml $CHCl_3$ and phospholipids with 300 ml CH_3OH. Further separation of nonpolar lipids was achieved by chromatography on Florisil, deactivated by 6% (w/v) water (150 g, 2.4 x 60 cm, column load ca. 6 g). The column was eluted with 200 ml hexane (Skellysolve B), 400 ml 5% ether in hexane, 600 ml 15% ether in hexane and 200 ml 25% ether in hexane. The fractions were monitored by thin layer chromatography (TLC) on Silica Gel H, developed with hexane-ether-acetic acid 65:35:1, sprayed with 10% sulfuric acid and charred at 150 C for 5 min. In this type of system, polyprenol esters run just ahead of cholesteryl esters, and free polyprenols run some distance ahead of cholesterol, at the level of free fatty acids (1).

Polyprenols esterified with fatty acids were eluted, together with cholesteryl esters, by 5% ether in hexane, while free polyprenols were eluted with triglycerides by 15% ether in hexane. Fractions containing esterified and free polyprenols were pooled separately and evaporated to dryness. The residues were hydrolyzed with 5-10% KOH in ethanol at 45 C for 3 hr, and the nonsaponifiable material recovered by extraction with petroleum ether. Fatty acids

TABLE I

Chemical Shifts of NMR Peaks of Polyprenols from Beef and Human Pituitaries[a]



2 *trans*, 15 *cis*

Proton assignments	δ, ppm CCl_4		δ, ppm C_6D_6
sec $C\underline{H}_3$	0.89 (d) J=6.2 Hz		0.86 (d)
$-C\underline{H}_2-CH-$	~1.26 (m)		
$-C\underline{H}_2-CH_2-OH$	~1.40 (m)		
d.b. $C\underline{H}_3$ *trans* terminal	1.58 (brs)	minor	1.57 (brs) ($1CH_3$)
d.b. $C\underline{H}_3$ *trans*	1.58 (brs)		1.61 (brs) ($2CH_3$)
d.b. $C\underline{H}_3$ *cis* terminal	1.66 (brs)	major	1.68 (brs) ($1CH_3$)
d.b. $C\underline{H}_3$ *cis*	1.66 (brs)		1.74 (brs) major
$=C-C\underline{H}_2-$	2.00 (brs)		2.16 (brs)
$=C-C\underline{H}_2-$	~2.00 (d) J=~3.5 Hz		~2.16 (brs)
$-CH_2-C\underline{H}_2-OH$	3.58 (t) J=6.5 Hz		3.45 (t)
d.b. $\underline{H}$	5.05 (m)		5.26 (m)

[a]Abbreviations used: sec = secondary, d.b. = double bond, br = broad, s = singlet, d = doublet, t = triplet, m = multiplet, J = coupling onstant in Hertz.

and glycerol were removed by washing with water.

The polyprenols were finally purified by preparative TLC on Silica Gel HF-254 plates (0.5 mm, 20 x 20 cm), developed in hexane-ether-acetic acid 65:35:1. They were detected under UV light (R_f 0.32) and eluted with ether. Traces of acetic acid were removed from the eluent by washing with water. Acetylation was carried out with acetic anhydride in pyridine.

Dolichol was isolated from pig liver, as described by Burgos et al. (2).

IR spectra were recorded on a Beckman IR-20A spectrophotometer. Solution spectra were taken in sodium chloride cells (0.1 mm).

NMR spectra were recorded on a Varian HA-100 spectometer, using CCl_4 or C_6D_6 as solvent and tetramethylsilane (TMS) as an internal reference standard.

Mass spectra were recorded on an MS-12 instrument, courtesy of A.M. Hogg, Department of Chemistry, The University of Alberta, Edmonton, Canada. The temperature of the probe was 300 C and the spectra were recorded at 70 eV with an accelerating voltage of 8000 V. The trap current was 100 μa.

RESULTS

Extraction of 600 g beef pituitaries (12 lots, 50 g each) yielded 20.8 g total lipids (3.5% of wet wt). Chromatography on acid-treated Florisil gave 6.2 g nonpolar lipids (30%) and 14.0 g phospholipids (67%). After further fractionation of the nonpolar lipids, 20 mg purified polyprenol was recovered from the polyprenol ester fraction and 58 mg from the free polyprenol fraction.

Extraction of 18 g human pituitaries (one lot, 38 pituitaries) yielded 712 mg total lipids (4% of wet wt). Chromatography on acid-treated Florisil gave 402 mg nonpolar lipids (56%) and 290 mg phospholipids (41%). The free polyprenol fraction from the nonpolar lipids yielded 25 mg purified polyprenol. Less than 10% of the polyprenol in human pituitary was esterified with fatty acids.

The chromatographic and spectroscopic properties of polyprenols from beef and human pituitaries were found to be essentially the same as those of authentic dolichol from pig liver.

The IR spectrum, determined in CCl_4 solution (~20 mg/0.1 ml), showed the presence of

free and bonded O-H stretching bands (3640 and 3400 cm^{-1}), C-H stretching of =C-H (3040 cm^{-1}), C-H stretching of $-CH_2-$ and $-CH_3$ (ca. 2970, 2930 and 2860 cm^{-1}), C=C stretching (1665 cm^{-1}), C-H bending of $-CH_2-$ and $-CH_3$ (1455 cm^{-1}), C-H bending of $-CH_3$ (1380 cm^{-1}), C-O stretching (1040 cm^{-1}) and C-H bending of trisubstituted double bond (840 cm^{-1}). Smaller bands were also present at 1320, 1240, 1130, 1090, 1065 and 890 cm^{-1}. The acetate displayed new strong bands at 1740 cm^{-1} and at 1235 cm^{-1} (C=O and C-O stretching), but no hydroxyl bands.

NMR spectra of the polyprenols from beef and human pituitaries were determined in CCl_4 and C_6D_6. Chemical shifts and assignment of protons are listed in Table I. Integration of the spectra indicated 16-18 *cis* isoprene residues and 2-3 *trans* isoprene residues.

Feeney and Hemming (3) suggested that dolichols contain two *trans* internal isoprene units, on the basis of integration measurements on the NMR spectrum in CCl_4 or C_6D_6 solution. This was confirmed in our studies by the following experiment: Decoupling of the double bond hydrogens by strong irradiation of their signal near 5.26 ppm in C_6D_6 causes the methyl signals between 1.5 and 1.8 ppm to become fairly sharp singlets (Fig. 1), and from this it is clearly seen that there can be only three *trans* methyl groups in dolichols (the one at 1.57 ppm being a terminal methyl) and hence only two internal *trans* isoprene units.

Mass spectra of the polyprenols from human and beef pituitaries each gave a molecular ion (M^+) peak at 1312, indicating that the main homologue had 19 isoprene units.

DISCUSSION

Results of these experiments indicate that the unknown lipid in beef and human pituitaries consists mainly of dolichol-19. Dolichols have previously been isolated from liver, spleen, pancreas, kidney, brain and skeletal muscle and also from algae, fungi, yeast and higher plants (2, 4-6). Our studies have shown that human pituitary is a particularly rich source, having a concentration at least 10 times that of pig liver, based on weight of wet tissue.

In bacteria, polyprenols in the form of phosphate derivatives have been found to play a role in biosynthesis of cell wall polymers, acting as intermediate acceptors in transfer of monosaccharides from nucleotide sugars to polysaccharides (5, 7-9). Dolichol phosphate appears to play a similar role in transfer of monosaccharides from nucleotide sugars to polysaccharides in liver (10), and there is also evidence that the polysaccharide acceptor is subsequently transferred to endogenous protein (11).

It seems probable, as suggested by Hemming (5), that phosphate derivatives are the biologically important form of polyprenols and that accumulation of free polyprenols is the result of hydrolytic activity. Whether this is the reason for the high concentration of polyprenol in the human pituitary or whether free polyprenols themselves also play an important biological role are questions which must await further experimental work.

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REFERENCES

1. Singh, H., and K.K. Carroll, Lipids 5:121 (1970).
2. Burgos, J., F.W. Hemming, J.F. Pennock and R.A. Morton, Biochem. J. 88:470 (1963).
3. Feeney, J., and F.W. Hemming, Anal. Biochem. 20:1 (1967).
4. Gough, D.P., and F.W. Hemming, Biochem. J. 118:163 (1970).
5. Hemming, F.W., Biochem. Soc. Symp. 29:105 (1970).
6. Richards, J.B., and F.W. Hemming, Biochem. J. 128:1345 (1972).
7. Osborn, M.J., Ann. Rev. Biochem. 38:501 (1969).
8. Rothfield, L., and D. Romeo, Bacteriol. Rev. 35:14 (1971).
9. Lennarz, W.J., and M.G. Scher, Biochim. Biophys. Acta 265:417 (1972).
10. Behrens, N.H., A.J. Parodi and L.F. Leloir, Proc. Nat. Acad. Sci. U.S. 68:2857 (1971).
11. Parodi, A.J., N.H. Behrens, L.F. Leloir and H. Carminatti, Ibid. 69:3268 (1972).

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Role of Luminal Lecithin in Intestinal Fat Absorption

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ABSTRACT

The effects of biliary lecithin on fat absorption were studied in 1 day bile fistula rats fed micellar solutions of bile salt, monoglyceride and radioactive free fatty acids. By electron microscopy and measurement of uptake of radioactivity into liver and adipose tissue, it was shown that in the absence of bile lecithin there was significant impairment of fat release from mucosa. Fat clearance was effected by the feeding of phosphatidyl choline or choline, but not phosphatidyl ethanolamine, inositol or cholesterol. In the absence of luminal choline there was a decrease in incorporation of radioactive leucine into mucosal protein. It is concluded that biliary and dietary lecithin or choline play an important role in triglyceride transport out of intestinal mucosa, by providing surfactant lecithin for the chylomicron envelope and by supporting mucosal protein biosynthesis.

INTRODUCTION

Previous studies from our laboratory have suggested that the synthesis and release of chylomicrons from isolated mucosal cells and everted sacs (1) is dependent on both protein and phospholipid biosynthesis. In the present experiments we have tested the role of lecithin synthesis in the formation and release of chylomicrons in vivo by interfering with the supply of the precursors of phosphatidyl choline that reach the intestinal mucosa from the lumen. For this purpose we compared uptake and clearance of fatty meals in normal and bile fistula rats supplemented with various phospholipids, choline and inositol. The results show that deprivation of dietary or biliary lecithin or choline leads to significant impairment of fat absorption. Since protein biosynthesis is also decreased under these conditions, it is not possible at the present time to dissociate the role of lecithin and protein biosynthesis in chylomicron formation and release. A preliminary account of this work has been presented (2).

MATERIALS AND METHODS

Materials

9,10[^{3}H]-Palmitic acid (specific activity 200/mc/mM) and 1[^{14}C]-leucine (specific activity 20 mc/mM) were obtained from New England Nuclear (Boston, Mass.). Monoolein, taurocholic acid and choline were purchased from Eastman Organic Chemicals (Rochester, N.Y.). Oleic and palmitic acids were obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio). Cholesterol was purchased from the British Drug Houses (Toronto); and inositol from the Sigma Chemical Co., (St. Louis, Mo.). Phosphatidyl choline (3) and phosphatidyl ethanolamine (4) were prepared from egg yolk.

Animal Experimentation

Male Wistar rats (300 g), which had been maintained on laboratory chow, were fasted 24 hr prior to experimentation. Bile fistula animals were prepared as follows. The animals were opened under ether anaesthesia and the bile duct was cannulated by inserting a thin polyethylene tube of suitable diameter. After passing the distal end of the cannula through a wound in the back of the animal, the abdomen was closed by suturing. Sham-operated animals were used as controls. After 1 day, the animals were fed by stomach tube, a micellar solution containing 800 mg of monoolein and free fatty acids (equimolar palmitic and oleic), in a 1:2 molar ratio. The monoolein and free fatty acids were prepared as a micellar solution by sonication with 1% taurocholic acid. For the radioactive experiments, 10 μc 9,10[^{3}H]-palmitate was added.

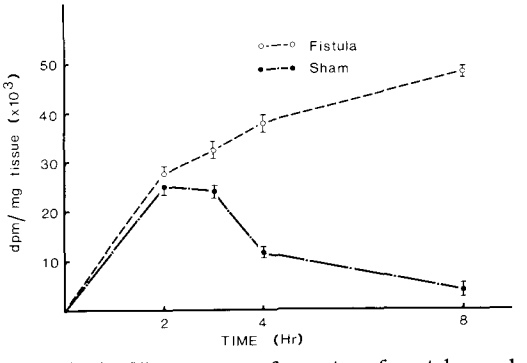

FIG. 1. Time course for rate of uptake and clearance of labeled lipid by rat jejuna. A suitable length of upper jejunum (6 in.) was taken for determination of radioactivity. Each point represents mean of three rats.

In Vitro Incubations

Everted sacs were prepared (5) from the jejuna of both fasted and fat-fed sham-operated and bile-fistula rats. The animals were sacrificed under ether anaesthesia, the jejuna excised, flushed with ice cold Krebs bicarbonate buffer, pH 7.4, everted and the mucosal side washed free of the luminal contents. Suitable lengths (5-10 cm) of the jejuna were allowed to fill with buffer, the ends tied and the sacs submitted to incubations as described in Table IV.

Assay of Protein Biosynthesis

Protein biosynthesis was assessed by incubating the everted sacs with radioactive leucine under the conditions described in Table IV. The reactions were stopped by the addition of 5 ml 10% trichloracetic acid containing 10 mg unlabeled L-leucine per milligram, and the suspensions homogenized. The protein precipitates were washed, extracted, and the radioactivity counted (6). The protein concentration was determined by the method of Hübscher et al. (7).

Lipid Analysis

Total lipid extracts were prepared according to the method of Folch et al. (8). The lipid extracts were resolved into fractions corresponding to triglycerides, free fatty acids, diglycerides, monoglycerides and phospholipids by thin layer chromatography on Silica Gel H (Merck & Co.) using heptane-isopropyl ether-acetic acid 60:40:3 v/v/v. The bands were detected by spraying with 0.05% 2,7-dichlorofluorescein in 50% methanol, and were visualized under UV light. Appropriate standards were used in all chromatographic separations. Lipid samples were extracted from gel scrapings with chloroform-methanol 2:1 v/v by partitioning with water at one-fifth the volume, including that of the sample. For measurement of radioactivity, fluorescein was removed by adding 4M NH_4OH as one-tenth the water volume. The chloroform layer was filtered through anhydrous Na_2SO_4 and a cotton plug into a scintillation vial, and dried at 40 C under nitrogen. To the dried residue was added 10 ml scintillation fluid, 0.5% 2,5-diphenyloxazole and 0.3% 1,4-bis(2-[5-phenyloxazole]) benzene (Amersham-Searle) in toluene. Radioactivity was measured in a Nuclear Chicago liquid-scintillation counter. Correction for quenching was made by the channels ratio method (9). The counting efficiency was 40%.

Electron Microscopy

Electron microscopy was performed as described previously (10).

RESULTS

Uptake and Clearance of Micellar Lipid in Normal and Fistula Rats

Figure 1 shows the rate of uptake and clearance of the fed radioactive lipid mixture by the jejuna of sham-operated and bile fistula rats. Almost all of the mucosal radioactivity was in the neutral fraction, less than 5% being found as free fatty acid. In the sham-operated animals a maximal concentration of label in the gut wall was reached ca. 2-3 hr after introducing the meal by stomach tube, after which it decreased and approached prefeeding levels by the end of a 6-8 hr period. This course of events corresponds to that noted by Gallagher et al. (11), who monitored the appearance of radioactivity in the lymph of normal rats after feeding a micellar solution of radioactive triolein. They found that the major part of the fat appeared in the lymph in the first 4 hr. In the bile fistula rats the initial uptake of radioactivity followed the course noted for the sham-operated controls. Instead of subsiding after the 3 hr period, the radioactivity in these animals continued to rise, although at a slower rate, and at the end of the 8 hr experimental period had reached twice the peak level of the intact rats. These results are also consistent with those of Gallagher et al. (11), who observed an abnormally low and delayed recovery of fat in the thoracic duct lymph of bile fistula rats. Clear-cut differences in fat absorption between control and bile fistula animals were also found recently by Rampone (12), who noted that the fistula group retained more of the label in the gut wall after 8 hr than the normal group and that there was less lipid recovered in the lymph.

Table I shows the distribution of radioactivity among the neutral lipid, free fatty acid and phospholipid fractions recovered from the gut wall during the course of the experiments. At no time did the free fatty acid level exceed 5% in the normal animals, which suggested that esterification of the monoglyceride was not impaired. In the fistual animals, the proportion of the free fatty acid was much higher. The highest amount of radioactivity in the free fatty acid fraction occurred in the bile fistula rats sacrificed at the 8 hr time interval. There was, however, no comparable increase in the level of monoglyceride at 8 hr. Dietschy (13) has claimed that mucosal triglyceride synthesis is normal in bile fistula rats, but this claim is based on rates of incorporation of fatty acid by tissue slices and may not reflect the present circumstances.

The impairment of clearance of mucosal fat by the bile fistula rat is further documented in

TABLE I

Per Cent Distribution of Radioactivity in Mucosal Lipids[a]

	Triglycerides		Diglycerides		Monoglycerides		Free Fatty Acids		Phospholipids	
Time, hr	Sham	Fistula	Sham	Fistula	Sham	Fistula	Sham	Fistula	Sham	Fistula
2	72 ± 4	69 ± 3	8 ± 2	11 ± 2	2 ± 0.5	3 ± 1	5 ± 1	11 ± 3	13 ± 2	6 ± 3
4	75 ± 3	72 ± 2	10 ± 1	9 ± 2	1 ± 0.5	2 ± 0.5	4 ± 2	13 ± 5	10 ± 2	4 ± 2
8	71 ± 4	63 ± 1	11 ± 3	14 ± 3	2 ± 1	1 ± 0.5	4 ± 2	18 ± 6	12 ± 3	4 ± 2

[a]Each figure represents the mean ±SEM of three animals. In each case a uniform portion of the jejunum (6 in.) was taken for determination.

Table II, which compares the uptake of radioactivity by adipose tissue and liver in the control and bile fistula animals. Although the radioactivities measured per gram of tissue do not allow the calculation of the actual amounts of fat absorbed by the two types of animal preparations, there remains little doubt that the bile fistula animal has suffered a severe impairment in its ability to produce or release chylomicrons, which could then be cleared by the liver and adipose tissue in the normal manner.

The above observations are supported by electron micrographic evidence. Micrographs prepared from the intestinal mucosa of a normal and bile fistula rat 2 hr after administration of the experimental meal, showed that both preparations contained large numbers of lipid particles, and there was little difference in the character of these particles between the two preparations. Clearly absent from the fistula preparations, however, were the extensive accretions of chylomicrons seen in the intercellular spaces of the mucosa of the control animal. Electron micrographs of the normal and the fistula rat 5 hr after the experimental meal showed that the normal animal had cleared most of the fat out of the mucosal cells by this time, as indicated by the absence of particles from the cell and chylomicrons from the intercellular spaces. There was little change in the appearance of the electron micrographs between the 2nd and 5th hr following the meal in the bile fistula animals, as would be expected from the analytical data presented above.

Effect of Phospholipids on Clearance of Mucosal Fat

In order to restore the mucosal formation and release of chylomicrons in the bile fistula rat, the experimental meals were supplemented with various bile lipid components or their precursors in amounts comparable to those supplied by normal bile flow over a 24 hr period. Since the experimental meals already included bile salts, which had no effect on the chylomicron release at least, this aspect of the bile composition was not further examined. Table III shows the effect of addition of egg yolk lecithin (50 mg) or choline (5 mg) on the amount of dietary fat retained by the mucosa of the bile fistula rat. For this comparison, total lipid extracts were prepared from the upper 6 in. of the jejunum 5 hr after the meal. In both instances the obtained clearance approached that noted for the sham-operated animals. Electron micrographs of the intestinal mucosa of the bile fistula rats that received lecithin or choline along with their fat meals, and were prepared 5 hr after the feeding, showed that there was an essentially complete disappearance of the fat particles from the mucosal cells.

TABLE II

Uptake of Radioactivity in Extrajejunal Tissues by Sham and Bile Fistula Rats[a]

	←dpm/g Liver→		←dpm/g Adipose tissue→	
Time, hr	Sham	Fistula	Sham	Fistula
2	12,200 ±1,900	960 ±400	1,300 ± 180	170 ±50
4	15,300 ±2,200	1,280 ±340	2,260 ± 340	190 ±40
8	16,100 ±2,300	1,310 ±400	3,180 ± 310	210 ±60

[a]Values are means ± SEM from six rats.

TABLE III

Fat in Jejunum after 5 Hours[a]

Preparation	Fat, mg
Sham	91 ± 18
Fistula	396 ± 32
Fistula + PC	82 ± 17
Fistula + choline	87 ± 22

[a]Each figure represents the mean ±SEM of six rats. A uniform portion (6 in.) of the upper jejunum was taken for determinations.

Figure 2 shows that inclusion of either lecithin or choline in the experimental meal led to a rapid appearance of radioactive palmitate in the adipose tissue and liver of the fistula rats, which approached the rates of uptake noted for the sham-operated animals. It is noteworthy that supplementation of the test meal with lecithin or free choline also led to a slightly increased uptake of radioactivity by the liver and adipose tissue of the sham-operated animals. This suggests that fat absorption may not normally proceed at maximum rates, unless dietary choline or lecithin is present. These observations suggest that lecithin, or at least some compound of choline, is intimately involved in chylomicron formation and release, presumably as a component of the lipoprotein envelope. This conclusion is in agreement with previous studies, which have established that much of both dietary and biliary lecithin is recovered in the chylomicron membrane during fat absorption.

The inclusion of egg yolk phosphatidyl ethanolamine (50 mg), cholesterol (1%) or inositol (5 mg) in the test meal had no effect on the clearance of fat from the intestine of the bile fistula rat (Fig. 3). This is effectively illustrated by the negligible amounts of radioactivity detected in the liver and adipose tissue of the fistula animals over the 8 hr examination period. This impression was supported by electron micrographs, which revealed fat laden cells in the intestinal mucosa of all the fistula animals 5 hr after feeding the above experimental meals. The addition of phosphatidyl ethanolamine, cholesterol and inositol to the meals of the sham-operated animals also failed to show any enhancement of the uptake of radioactivity by the liver or adipose tissue. The lack of promotion of mucosal clearance by phosphatidyl ethanolamine would be expected, if the effect is specific for lecithin, since it has been already reported (14) that the intestinal mucosa lacks the ability to convert phosphatidyl ethanolamine into phosphatidyl choline via methylation. The lack of effect of inositol on the mucosal clearance of lipid is in contrast to its effect in liver, where inositol is known to act as a lipotropic agent (15). It is possible that the amounts of cholesterol required for chylomicron formation and secretion are probably already available to the intestine, since it has been reported that cholesterogenesis increases eight-fold in biliary diversion (13). In any event, the amounts of phosphatidyl ethanolamine, phosphatidyl inositol and cholesterol found in the chylomicron membrane are rather small in comparison to the content of lecithin.

Effect of Phospholipids on Biosynthesis of Mucosal Protein

Table IV gives the results of the incorporation of radioactive leucine into the mucosal proteins of everted sacs from fasted and fat-fed normal and bile fistula animals. It is seen that the specific activities of the mucosal protein are ca. 30% lower in the fasted bile fistula rats, and that this difference increases to ca. 44%, when comparisons are made in the fed animals. It is possible that the synthesis of the secretory lipoproteins is inhibited even more completely than the analysis of the specific activities of the total protein would indicate. This could not be

TABLE IV

Effect of Phospholipids on Biosynthesis of Mucosal Proteins[a]

Preparation	Fasted animal	Fed animal
Sham	5,180 ± 340	14,870 ± 990
Sham + PC	6,330 ± 780	18,680 ± 1,240
Sham + Choline	6,690 ± 810	18,190 ± 1,380
Fistula	2,670 ± 310	9,260 ± 640
Fistula + PC	6,750 ± 840	17,230 ± 1,760
Fistula + Choline	7,190 ± 680	18,470 ± 1,590

[a]Everted sacs were prepared from jejuna as described in Methods. Incubation flasks contained everted sacs in Krebs-bicarbonate buffer, pH 7.4 plus ^{14}C-leucine (2×10^6 dpm). Reactions were carried out for 1 hr at 37 C in a Dubnoff metabolic shaker. Lecithin (50 mg) and choline (5 mg) were fed to animals as before and animals then sacrificed after 3 hr. Results represent mean of three experiments.

immediately ascertained, because the chylomicron proteins cannot be readily isolated in the absence of significant chylomicron release. The above results suggest that synthesis of protein in the normal fed animals is increased over that seen in the normal fasted animal and that the bile fistula preparation is unable to supply the increased needs for protein during fat absorption, when either choline or lecithin is lacking in the diet or in the bile. Addition of lecithin or choline resulted in a restoration of the leucine incorporation to the levels noted in the sham-operated animals. Furthermore, the effect was more pronounced in the fistula animals that had received the experimental fat meal than in the fasting animals.

The latter experiments suggest that the effect of lecithin or choline upon chylomicron formation and release is at least partially dependent upon an effective synthesis of secretory or membrane proteins, which accompany the fat globules as they leave the cell. This interpretation of the results is in agreement with the findings of Mookerjea (16), who claimed that the triglyceride accumulation in the livers of choline-deficient rats is due to an impairment in the biosynthesis of the lipoproteins responsible for the transport of triglycerides out of the liver. Choline supplementation brought about an immediate release of the block in protein synthesis and secretion.

DISCUSSION

From the results obtained, by both electron microscopy and measurement of uptake of radioactivity by extrajejunal tissues, it is obvious that the absence of both dietary and biliary lecithin results in significant impairment of fat clearance from rat intestinal mucosa. Addition of either lecithin or choline caused a release of fat at a rate comparable to that of the control animals. To our knowledge, this is the first report that demonstrates a role for biliary lecithin in fat absorption.

A function for lecithin in fat absorption was proposed in earlier studies. Sinclair (17) first suggested, in 1929, that a phospholipid was an obligatory intermediate in the absorption of fat. Frazer (18,19) claimed that, in the absence of dietary choline, rats fed triglyceride accumulated fat in the intestinal mucosa, and that addition of dietary choline markedly enhanced fat clearance. Later, Augur et al. (20) reported that lecithin increased the digestibility and absorption of fat in man, while Tidwell (21) concluded that by counting chylomicra he could demonstrate a small increase in the rate of absorption when free choline is added to a

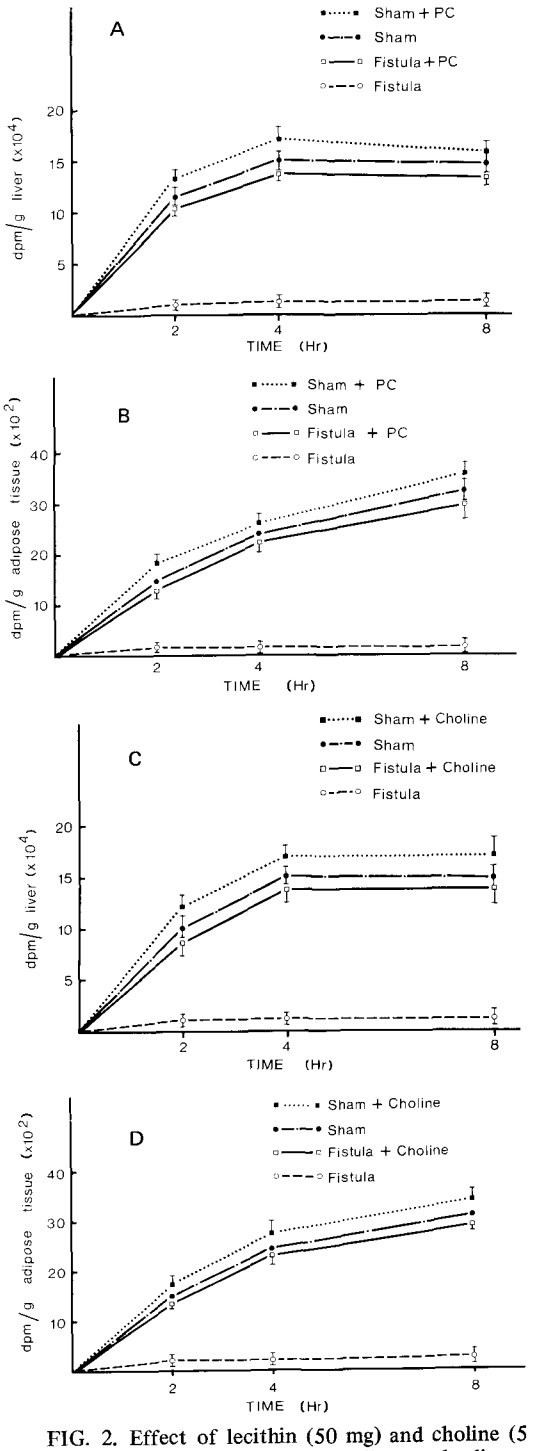

FIG. 2. Effect of lecithin (50 mg) and choline (5 mg) on uptake of labeled lipid by liver and adipose tissue. A and B show effect of lecithin on uptake by liver and adipose tissue, respectively. C and D show effect of choline. Each point represents mean of six rats. Vertical bars are standard error of mean.

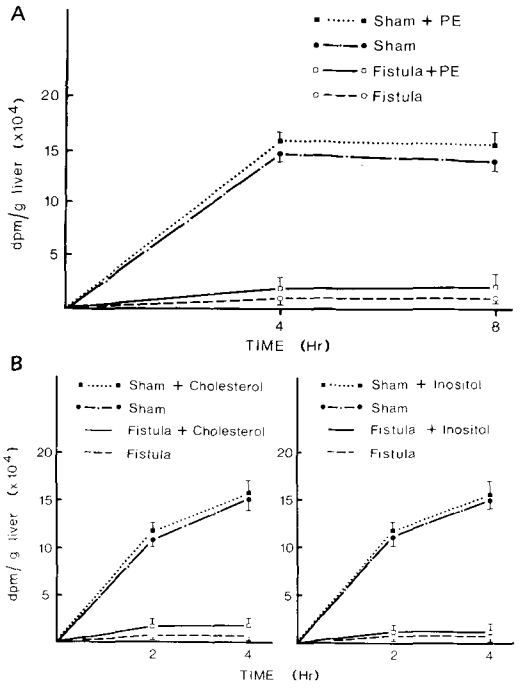

FIG. 3. Effect of phosphatidyl ethanolamine (50 mg), cholesterol (1%) and inositol (5 mg) on uptake of labeled fat by liver. A shows effect of phosphatidyl ethanolamine on uptake by liver. B shows effect of cholesterol and inositol. Each point represents mean of six rats. Vertical bars are standard error of mean. Uptake of labeled fat by adipose tissue showed a similar pattern.

fat meal. Tasker and Hartroft (22), using morphological techniques, were unable to confirm the findings of either Frazer or Tidwell. In addition, Zilversmit et al. (23), using [^{32}P], found that the turnover of mucosal phospholipids was low, which presumably ruled out their participation as intermediates in fat absorption. It could not rule out a limited synthesis of lecithin necessary for chylomicron formation, much of which could proceed normally via the lysolecithin pathway.

Of the phospholipids in rat chylomicrons, 70-80% is lecithin (24). The fatty acid composition of chylomicron lecithin has been shown to be rather constant and largely independent of the composition of the fatty meal given (25). In addition, Scow et al. (26) have concluded that a maximum of 40% of chylomicron lecithin was derived from dietary sources, even when the concentration of lecithin in the diet was two to three times that in the chylomicrons. Of the 60% or more of the chylomicron lecithin which therefore came from endogenous sources, the bile would probably be a major contributor. If lecithin is important in the total absorption process, one would expect an increase in its synthesis during absorption. Indeed Hübscher et al. (27) found that, of all the phospholipids of the intestinal mucosa, only lecithin showed a five-fold increase in specific activity during fat absorption, as compared with carbohydrate-fed controls.

It is possible that the various components of the bile have a distinct function in the different stages of fat absorption. The bile acids appear to aid absorption of monoglycerides and free fatty acids into the intestinal mucosa, while the lecithin moiety may be involved in secretion out of the intestine. This can be deduced from the present work along with the earlier studies of Morgan and Börgstrom (28) who showed a marked impairment in absorption into the intestine, if micellar solutions of dietary glycerides were made with phospholipids and not bile salts. Rampone (12) has recently found, both in experiments where bile fistula rats were perfused with a micellar solution of bile salt, monoglyceride and free fatty acid, and in experiments using everted sacs from small intestine of bile fistula rats, that absorbed lipid accumulated in the intestine. He concluded that bile contained one or more components that played a role in the phase of absorption involved with transport out of the mucosa. From the present studies, one such component would appear to be lecithin. Morgan (29) had previously claimed little difference in the lymphatic output of esterified fat between normal and bile fistula rats. In view of the present data, the lack of effect by biliary diversion observed by Morgan must have been due to lecithin which was present at the level of 1.2% in the commercial emulsion.

The importance of phospholipid in fat clearance can also be inferred from the work of Friedman and Cardell (30), who have reported the effects of puromycin on the structure of the intestinal mucosa during fat absorption. Puromycin treatment caused the absorptive cells to accumulate increased quantities of lipid that were devoid of membrane during fat absorption, and, in addition, puromycin-treated cells contained much less rough endoplasmic reticulum and Golgi membranes. When such membranes were absent, the cell's ability to discharge chylomicra was impaired and lipid accumulated. The findings of Friedman and Cardell are in agreement with some biochemical evidence for inhibition of phospholipid synthesis by puromycin (1). In addition, Van den Berg and Hulsmann (31) have reported that even lack of the appropriate diglyceride lowered lecithin synthesis in the gut and that this

impaired chylomicron synthesis and release. Since phospholipid is an integral part of membranes, its biosynthesis must therefore be essential for membrane formation.

It is also obvious that the presence of lecithin or choline supports or stimulates mucosal protein synthesis. This role of lecithin or choline in promoting protein synthesis in the gut is similar to the effect of phosphoryl choline in promoting protein synthesis in livers of normal and choline-deficient rats (32-33). The exact mechanisms by which biliary lecithin is involved in the formation and release of chylomicrons and the nature of its involvement in the synthesis of chylomicron protein must await further investigation.

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REFERENCES

1. O'Doherty, P.J.A., I.M. Yousef and A. Kuksis, Fed. Proc. 31:2739 (1972).
2. O'Doherty, P.J.A., G. Kakis and A. Kuksis, Proc. Can. Fed. Biol. Soc. 15:235 (1972).
3. Kuksis, A., and L. Marai, Lipids 2:217 (1967).
4. Holub, B.J., and A. Kuksis, Ibid. 4:466 (1969).
5. Wilson, T.H., and G. Wiseman, J. Physiol. 123:116 (1954).
6. Villa-Trevino, S., K.H. Shull and E. Farber, J. Biol. Chem. 238:1757 (1963).
7. Hübscher, G., G.R. West and D.N. Brindley, Biochem. J. 97:629 (1965).
8. Folch, J., M. Lees and G.H. Sloane-Stanley, J. Biol. Chem. 226:497 (1957).
9. Baillie, L.A., Int. J. Appl. Rad. Iso. 8:1 (1960).
10. Yousef, I.M., and A. Kuksis, Lipids 7:380 (1972).
11. Gallagher, N., J. Webb and A.M. Dawson, Clin. Sci. 29:73 (1965).
12. Rampone, A.J., J. Physiol. 222:679 (1972).
13. Dietschy, J.M., Fed. Proc. 26:1589 (1967).
14. Wise, E.M., and D. Elwyn, J. Biol. Chem. 240:1537 (1965).
15. Beveridge, J.M.R., Science 99:539 (1944).
16. Mookerjea, S., Can. J. Physiol. Pharmacol. 46:499 (1968).
17. Sinclair, R.G., J. Biol. Chem. 82:117 (1929).
18. Frazer, A.C., Nature 157:414 (1946).
19. Frazer, A.C., Arch. Sci. Physiol. 2:15 (1948).
20. Augur, V., H.S. Rollman and H.J. Deuel, Jr., J. Nutr. 33:177 (1947).
21. Tidwell, H.C., J. Biol. Chem. 182:405 (1950).
22. Tasker, R.R., and W.S. Hartroft, Nature 164:155 (1949).
23. Zilversmit, D.B., I.L. Chaikoff and C. Entenman, J. Biol. Chem. 172:637 (1948).
24. Zilversmit, D.B., J. Clin. Invest. 44:1610 (1965).
25. Nilsson, A., Biochim. Biophys. Acta 152:379 (1968).
26. Scow, R.O., Y. Stein and O. Stein, J. Biol. Chem. 242:4919 (1967).
27. Hübscher, G., M.E. Smith and M.I. Gurr, in "Metabolism and Physiological Significance of Lipids," Edited by R.M.C. Dawson and D.H. Rhodes, John Wiley and Sons, Inc., London, 1964, p. 229.
28. Morgan, R.G.H., and B. Börgstrom, Q. J. Exp. Physiol. 54:228 (1969).
29. Morgan, R.G.H., Ibid. 49:457 (1964).
30. Friedman, H.I., and R.R. Cardell, Jr., J. Cell. Biol. 52:15 (1972).
31. Van den Berg, J.W.O., and W.C. Hulsmann, "Abstracts of the 7th Meeting, Federation of European Biochemical Societies," Vol. 7, Varna, Bulgaria, 1971, p. 210.
32. Mookerjea, S., Fed. Proc. 30:143 (1971).
33. Mookerjea, S., and E. Marai, J. Biol. Chem. 246:3008 (1971).

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Monoethylenic Isomers in Cardiac Lipids of Rats Fed Partially Hydrogenated Herring Oil[1]

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ABSTRACT

Compositional studies have been carried out to compare the monoethylenic fatty acid isomers of a partially hydrogenated herring oil with those found in the cardiac lipid of young rats fed this oil for 1 or 16 weeks. In general, all geometrical and positional isomers with chain lengths C_{16}, C_{18}, C_{20} and C_{22} found in the hydrogenated oil were also observed in cardiac lipid. Evidence was also obtained for the occurrence of β-oxidation in the catabolism of the *cis* and *trans* isomers of these long chain acids.

INTRODUCTION

In the past few years nutritional studies have demonstrated that the feeding of partially hydrogenated oils containing C_{22} fatty acids to young rats results in increased deposition of cardiac fatty acids (1,2). These oils have been shown to contain numerous geometrical and positional monoethylenic isomers (3,4). Little, however, is known of the isomers in the deposited lipid in relation to those present in the dietary oil. To gain this information, compositional studies have been carried out on a partially hydrogenated herring oil and on the cardiac lipids of young rats fed this oil for 1 week (period of maximum lipid deposition) or for 16 weeks.

EXPERIMENTAL PROCEDURES

Male weanling rats were fed a test diet containing 20% oil, comprising partially hydrogenated herring oil and liquid corn oil (3:1). At 1 and 16 weeks hearts were removed and extracted for lipids. Details of these procedures have been described previously (2).

Pooled lipids from five hearts at each time interval were converted to methyl esters with sulfuric acid-methanol-benzene and analyzed by gas liquid chromatography (GLC). Methyl esters were separated into fractions according to chain length by preparative GLC. Fractions were subsequently studied by GLC, separated by silver ion thin layer chromatography, and positional isomers determined by micro-ozonolysis. These techniques were as described previously (3).

RESULTS

The fatty acid compositions, in terms of chain length, of the cardiac lipids of control and experimental animals are shown in Table I. These data are in accordance with previous

[1]Presented at the AOCS Meeting, Ottawa, September 1972.

TABLE I

Fatty Acid Composition of Control and Experimental Cardiac Lipids at 1 and 16 Weeks

	Area % composition[a]			
	1 Week		16 Weeks	
Fraction[b]	Control[c]	Experimental	Control	Experimental
14	0.3	1.3	0.3	0.8
16	16.1	11.7	13.2	12.0
18	48.0	40.0	51.9	51.3
20	21.3 (1.0)[d]	24.7 (13.5)	23.3 (1.9)	27.1 (5.3)
22	14.2 (-)	22.3 (15.7)	11.3 (0.4)	8.8 (3.1)
Fatty acids, mg/g heart				
mg/g heart	19.4	48.6	16.2	19.2

[a]SE30 column.

[b]Fractions according to chain lengths. Small amounts of odd and branched chain acids are incorporated with previous major fraction.

[c]Control animals were fed a similar diet containing 20% lard and corn oil (3:1).

[d]Figures in parentheses refer to per cent monoene of total methyl esters. These data were obtained on a DEGS column after preparative gas liquid chromatography.

TABLE II

Monoethylenic Isomers in Partially Hydrogenated Herring Oil and Cardiac Lipids of Experimental Rats at 1 and 16 Weeks

Chain length	Lipid source	Composition of aldehyde-esters, mol%										
		5	6	7	8	9	10	11	12	13	14	15
16	HHO[a] (7.5%)[b]											
	trans (37%)[c,d]	1	12	4	14	45	14	6	3	1	—	—
	cis (63%)	—	2	3	5	80	5	4	1	—	—	—
	1W (1.0%)											
	trans (53%)	22	11	5	20	26	7	6	2	1	—	—
	cis (47%)	—	6	12	8	61	5	4	2	2	—	—
	16W (2.7%)											
	trans (55%)	14	9	6	13	34	12	6	4	2	—	—
	cis (45%)	3	3	11	5	66	5	4	2	1	—	—
18	HHO (13.1%)											
	trans (35%)	—	—	5	11	40	17	17	7	3	—	—
	cis (65%)	—	—	6	3	66	3	16	3	3	—	—
	1W (15.1%)											
	trans (23%)	6	3	11	10	39	7	12	6	3	3	—
	cis (77%)	—	—	11	4	63	2	18	1	1	—	—
	16W (16.7%)											
	trans (34%)	4	3	14	8	41	5	10	8	3	4	—
	cis (66%)	—	—	13	2	57	1	25	1	1	—	—
20	HHO (19.9%)											
	trans (33%)	—	—	—	3	9	15	50	15	5	2	1
	cis (67%)	—	—	—	1	10	4	75	5	5	—	—
	1W (13.5%)											
	trans (20%)	—	—	4	4	25	16	32	10	6	3	—
	cis (80%)	—	—	1	2	25	4	56	4	7	—	—
	16W (5.3%)											
	trans (19%)	—	—	2	3	14	14	40	16	7	4	—
	cis (81%)	—	—	—	2	28	3	56	4	8	—	—
22	HHO (29.0%)											
	trans (32%)	—	—	—	—	2	15	57	17	6	2	1
	cis (68%)	—	—	—	—	2	3	82	4	9	—	—
	1W (15.7%)											
	trans (27%)	—	—	—	—	6	12	56	15	9	2	—
	cis (73%)	—	—	—	—	3	4	83	4	6	—	—
	16W (3.1%)											
	trans (26%)	—	—	—	—	5	15	52	16	10	2	—
	cis (74%)	—	—	—	—	3	5	80	4	8	—	—

[a]HHO refers to partially hydrogenated herring oil; 1W and 16W refer to cardiac lipids at 1 and 16 weeks, respectively.

[b]Figures in parentheses after HHO, 1W and 16W refer to percentage of monoene in total methyl esters.

[c]Figures in parentheses after *cis* and *trans* refer to per cent within monoene.

[d]All silver ion thin layer chromatography separation and ozonolysis reactions were carried out in duplicate, except with fraction 16 where insufficient material was available.

observations (1,2), in that they show the increased deposition of cardiac fatty acids in experimental animals (48.6 mg/g heart) compared to controls (19.4 mg/g heart) at 1 week; whereas at 16 weeks values for experimental animals approach those of the controls. Also both C_{20} and C_{22} monoenes show relatively high values at 1 week and fall off to lower levels at 16 weeks. At both time periods, however, these values are appreciably higher than corresponding controls.

Table II summarizes the monoethylenic isomers found in the dietary oil and in the cardiac lipids at both time periods, for the C_{16}, C_{18}, C_{20} and C_{22} chain lengths.

In the C_{22} monoenes, all major isomers present in the dietary oil can be observed in the cardiac fatty acids at 1 and 16 weeks. Further, although there is considerable difference in the amounts of monoene involved at 1 and 16 weeks, the relative proportions of positional isomers within the *cis* series are similar at these two periods and are comparable to proportions observed in the original oil. Similar considerations apply to relative proportions of positional isomers within the *trans* series. These observations demonstrate that within each geometric series all isomers are similarly deposited. The fact that *trans* percentages are similar at 1 (27%) and 16 weeks (26%) but are lower than

that (32%) in the dietary oil does, however, indicate differences between geometrical isomers.

With the C_{20} monoenoic isomers, again, *trans* percentages are similar at 1 and 16 weeks but are lower than that in the oil fed. A major point of interest in these data is the increase in proportion of the C_{20} $\Delta 9$ isomers in both geometric series at both time intervals. These isomers probably derive from β-oxidation of the major C_{22} $\Delta 11$ isomers. The C_{20} $\Delta 7$ isomers, which were not found in the original oil, would be derived in a similar manner from the C_{22} $\Delta 9$ isomers.

In the octadecenoic and hexadecenoic isomers, a continuation of this β-oxidation sequence can be observed by the increase in proportion of the C_{18} $\Delta 7$ and C_{16} $\Delta 5$ isomers, respectively. With the C_{16} $\Delta 5$ isomers, however, it should be noted that the increase in proportion in the *trans* series appears greater than that in the *cis* series. Similar considerations hold for the C_{18} $\Delta 5$ isomers.

In both the C_{18} and C_{16} chain lengths, the relative deposition of geometric isomers is considerably different from that observed in the C_{20} and C_{22} chain lengths. In the C_{18} isomers there is an initial drop in *trans* percentage at 1 week, as was observed with the longer chain fractions. However at 16 weeks the *trans* percentage increases to a level similar to that in the dietary oil. In the C_{16} isomers *trans* percentages are similar at 1 and 16 weeks, but in this case are higher than in the herring oil.

DISCUSSION

From the present results it would appear that, on a qualitative basis, most of the monoethylenic isomers present in the chain lengths C_{16}-C_{22} in the dietary oil are also found in cardiac lipid. Also, in the docosenoic isomers, all isomers within each geometric series are similarly deposited. The relative proportions of *cis* and *trans* isomers, however, appear to vary throughout the chain lengths C_{16}-C_{22}, giving no consistent pattern, and are a probable consequence of differences in absorption as well as metabolism.

The results do provide evidence for the role of β-oxidation in the catabolism of dietary docosenoic acids in both their *cis* and *trans* forms: C_{22} $\Delta 11 \rightarrow C_{20}$ $\Delta 9 \rightarrow C_{18}$ $\Delta 7 \rightarrow C_{16}$ $\Delta 5$.

This confirms and extends the work of Craig and Beare (5) and Carreau et al. (6), who presented evidence for the β-oxidation in rats of the *cis* isomers of C_{22} $\Delta 11$ and C_{22} $\Delta 13$, respectively. The former authors (5) were unable to detect the C_{16} $\Delta 5$ *cis* isomer in their study, and the present results would tend to support this observation since only a small proportion is observed. However the C_{16} $\Delta 5$ *trans* isomer is found in the present study as a significant proportion of the C_{16} *trans* isomers. It is interesting to speculate that this isomer may represent a block in the β-oxidation sequence similar to that described by Willebrands and Van der Veen in their studies on the metabolism of elaidic acid in the perfused rat heart (7). These authors reported an accumulation of the C_{12} $\Delta 5$ *trans* isomer and speculated that the α,β dehydrogenation step was perhaps inhibited in the case of $\Delta 5$ *trans* isomers by steric hindrance.

Similar considerations would apply to the C_{18} $\Delta 5$ *trans* isomer, which would derive from C_{22} $\Delta 9$ *trans* isomer after two cycles of β-oxidation.

REFERENCES

1. Beare-Rogers, J.L., E.A. Nera and H.A. Heggtveit, Can. Inst. Food Technol. 4(3):120 (1971).
2. Beare-Rogers, J.L., and E.A. Nera, Lipids 7:548 (1972).
3. Conacher, H.B.S., and B.D. Page, JAOCS 49:283 (1972).
4. Conacher, H.B.S., B.D. Page and R.K. Chadha, Ibid. 49:520 (1972).
5. Craig, B.M., and J.L. Beare, Can. J. Biochem. 45:1075 (1972).
6. Carreau, J.P., A. Thoron, D. Lapous and J. Raulin, Bull. Soc. Chim. Biol. 50:1973 (1968).
7. Willebrands, A.F., and K.J. Van der Veen, Biochim. Biophys. Acta 116:583 (1966).

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Application of Silica Gel-Sintered Plate to Thin Layer Lipid Chromatographic Analysis[1,2]

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ABSTRACT

The merits and disadvantages of using the silica gel-sintered plate for lipid chromatographic analysis were investigated in detail. The commercially available sintered plate could be used repeatedly, employing the reconditioning procedure which involved chromic-sulfuric acid treatment and subsequent activation. The reconditioned sintered plate has now been used successfully 20 times, for lipid analysis without any deterioration of the excellent resolution power for complex lipid mixtures for all the solvent systems. Since the sintered plate is sturdy, the chromatoplate could be immersed directly in the liquid reaction mixture, so that spots on the chromatogram could be seen and impregnated plates prepared simply. The sintered plate was found to be much more economical than the silica gel-coated plate.

INTRODUCTION

TLC using silica gel coatings has been an essential tool in lipid analysis, and the tech-

[1]Presented in part at the Japan Oil Chemists' Society 20th Annual Meeting, Tokyo, November 1972.

[2]The following abbreviations are used in this paper: TLC, thin layer chromatography; PA, phosphatidic acid; PS, phosphatidylserine; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PI, phosphatidylinositol; SPM, sphingomyelin; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; CMH, ceramide-monohexoside; CDH, ceramide-dihexoside; CTH, ceramide-trihexoside; NL, neutral lipid.

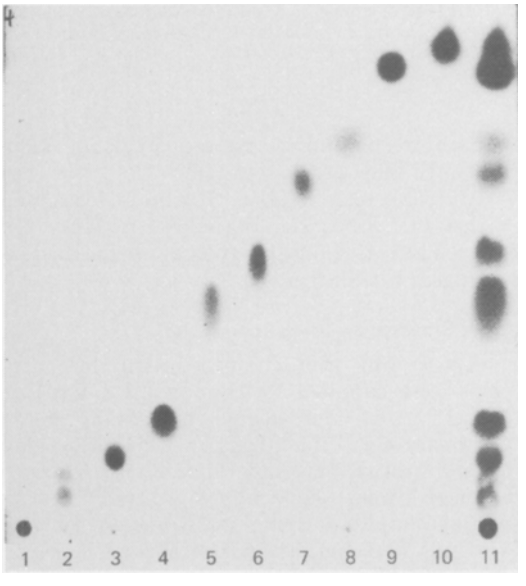

FIG. 1. Separation of reference neutral lipids on washed sintered plate. Chromatogram developed with system I. Detection of spots: charring with chromic-sulfuric acid spray. 1, 1-Monostearin; 2, diglycerides (upper spot 1,3-dipalmitin, lower spot 1,2-dipalmitin); 3, cholesterol; 4, cetyl alcohol; 5, stearic acid; 6, triglycerides (prepared from olive oil); 7, diacyl glyceryl ether (1,2-dipalmitoyl 3-octadecyl glycerol); 8, fatty acid methyl esters (methanolysis products from egg yolk phospholipids); 9, cholesteryl palmitate; 10, squalane; 11, mixture of 1-10.

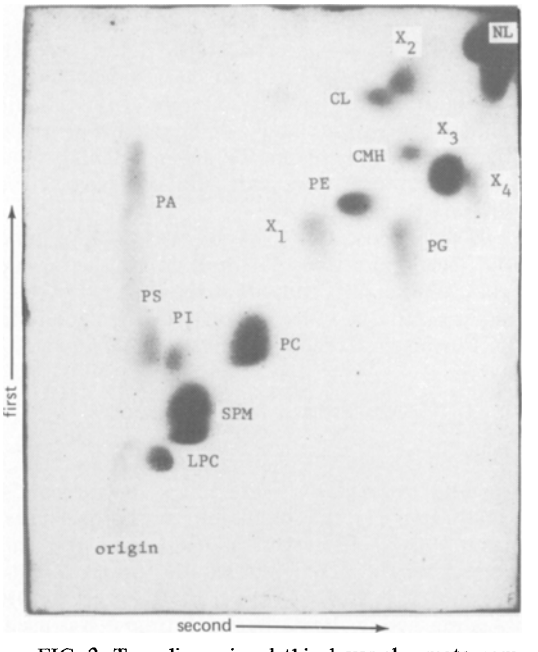

FIG. 2. Two dimensional thin layer chromatogram of complex mixture of total lipid extract from yeast cells *(Tricosporone cutanium KC 4-3)* supplemented by LPC, SPM, PG and CMH on washed sintered plate. Chromatogram developed first with solvent system VI and then with solvent system VII. Detection of spots: charring with molybdenum blue spray. X_1, X_2, X_3 and X_4 are unidentified lipids.

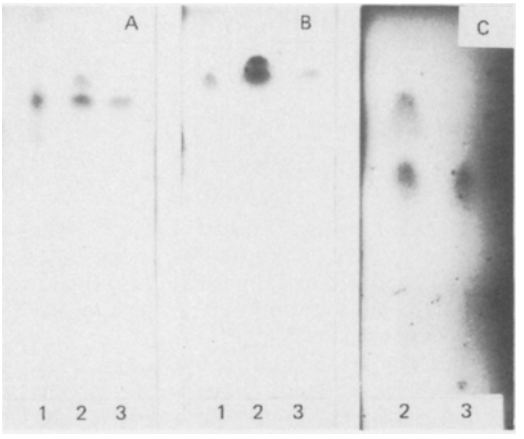

FIG. 3. Separation of mixture of cardiolipin and unidentified phospholipid, P(X), obtained from yeast cells on unwashed sintered plate. Solvent systems: (a)= (IX), (b) = (X), (c) = (VI). Detection of spots: charring with molybdenum blue spray. 1, PA; 2, CL + P(X); 3, CL.

nique has been steadily improved with many modifications (1-3).

Recently a new plate for TLC, made from a silica gel-fused glass powder mixture, has been developed and sold commercially under the trade name of "Replate" (4).

The silica gel-sintered plate should theoretically have a number of advantages over the old coatings: it does not contain any organic binders, is sturdy, and can be used repeatedly by soaking the chromatogram in cleaning solution—therefore new plates do not have to be prepared.

The purpose of this study was to determine optimum conditions for lipid analysis using the plate. Our results show that the sintered plate is superior to the silica gel-coated plate for lipid TLC analysis, except for preparative TLC.

MATERIALS AND METHODS

Plate

Silica gel-sintered plate: 26 x 76 mm and 50 x 200 mm plates (commercially available sizes) and 100 x 125 mm plates (made for this experiment) were purchased from Yamato Kagaku Co., Ltd. (Tokyo). Each sintered plate was marked in the corner each time it was used. Sintered plates were reconditioned by immersing in chromic-sulfuric acid cleaning solution (saturated $K_2Cr_2O_7$ aqueous solution-concentrated H_2SO_4 1:1 v/v) overnight and then washing in tap water for 1 hr. Finally they were washed three times with distilled water, activated at 120 C for 2 hr, and stored in a desiccator containing anhydrous calcium chloride until they were next used.

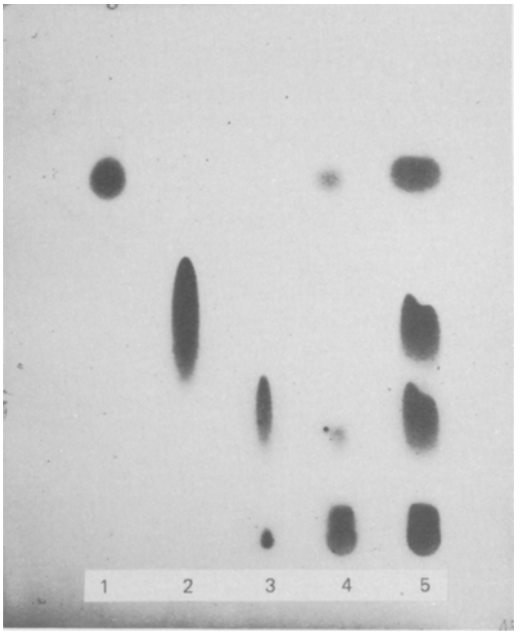

FIG. 4. Thin layer chromatogram of fatty acid methyl esters on argentation-sintered plate. Chromatogram developed with petroleum ether-diethyl ether 95:5 v/v. Detection of spots: charring with chromic-sulfuric acid spray. 1, Methyl stearate; 2, methyl elaidate; 3, methyl oleate; 4, methanolysis products from yeast lipid *(Tricosporone cutanium KC 4-3)*; 5, mixture of 1-4.

Silica gel plate: 100 x 150 mm plates coated with either Silica Gel H (Merck) or Wako Gel B-5 (Wako Chemicals, Tokyo) were used as standards for comparison.

Lipid Samples

C_8 to C_{18} even-numbered saturated fatty acids were supplied by Nippon Oil & Fat Co., Ltd. (Tokyo), and methyl elaidate by Nihon Chromato Co., Ltd. (Tokyo). Dipalmitin, cholesteryl palmitate, and dipalmitoyl glyceryl ether (5) were chemically synthesized. Other reference neutral lipids and some fat soluble vitamins were purchased from Wako Chemicals. PC, LPC, PE and SPM were prepared from egg yolk (6); PS and PI were purified from beef brain and yeast cellular lipids, respectively (7). CL was obtained from T. Takahashi (Institute of Kitasato, Tokyo). Dipalmitoyl-PC was obtained from Sigma Chemical Co., Ltd. (London), and PG from Serdary Research Labs. (Ontario). Each phospholipid was homogeneous on thin layer chromatograms. CMH, CDH and CTH were obtained from S. Ando (Dept. of

Biochemistry, Tokyo Metropolitan Institute for Gerontology). Total lipid extracts from yeast cells were obtained as described previously (8).

Chemicals

Organic solvents used for the analysis were further purified by distillation in an all-glass apparatus. Other organic and inorganic reagents, of analytical grade or the highest quality commercially available, were used without further purification.

Industrial grade potassium dichromate and concentrated sulfuric acid were used for cleaning the used sintered plates.

Developing Systems

The following solvent mixtures were used: (I) petroleum ether-diethyl ether-glacial acetic acid 90:10:1 v/v; (II) petroleum ether-diethyl ether-glacial acetic acid 50:50:1 v/v; (III) *n*-hexane; (IV) *n*-hexane-benzene 1:1 v/v; (V) cyclohexane-diethyl ether 8:1 v/v; (VI) chloroform-methanol-water 65:25:4 v/v; (VII) chloroform-methanol-concentrated ammonia 65:35:5 v/v; (VIII) *n*-butanol-glacial acetic acid-water 60:20:20 v/v; (IX) diisobutylketone-glacial acetic acid-water 45:25:5 v/v; and (X) chloroform-methanol-acetone-glacial acetic acid-water 5:1:2:1:0.5 v/v.

Detecting Reagents

The following detecting reagents were used: chromic-sulfuric acid, concentrated sulfuric acid, iodine vapor, 2',7'-dichlorofluorescein, phosphomolybdic acid, oil red (9) and Sudan black staining reagent (10) (above for nonspecific detection of lipids), antimony trichloride (for sterols), molybdenum blue (for phospholipids) (11), chlorox-benzidine (for amide linkage) (12), orcinol and anthrone (for glycolipids), ninhydrin (for free amino groups), Dragendorff reagent (for choline) and Levine-Chargaff reagent (for choline staining) (13).

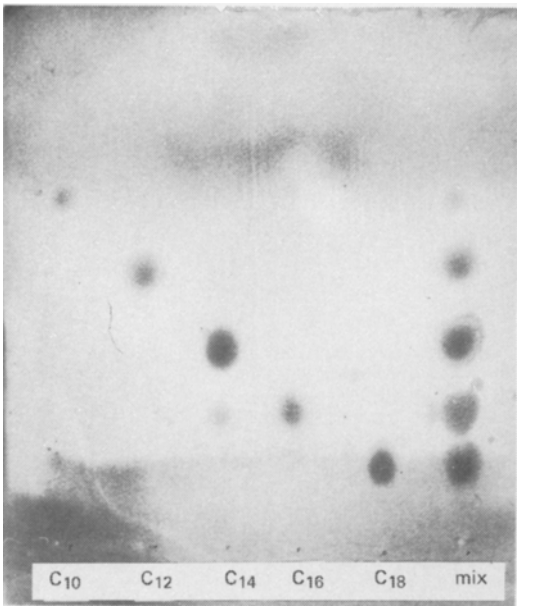

FIG. 5. Separation of C_{10}-C_{18} saturated fatty acids by reversed phase partition chromatography on sintered plate. Stationary phase: kerosene. Solvent system: 90% acetic acid (saturated with kerosene). Detection of spots: dyeing by lead acetate-ammonium sulfide reagent.

RESULTS AND DISCUSSION

A typical chromatogram of neutral lipids (each sample 1-10 μg) on a sintered plate washed and activated by the cleaning and reconditioning procedure is shown in Figure 1. The washed sintered plate gave excellent resolution for neutral lipids mixtures containing fat soluble vitamins and hydrocarbons using all the solvent systems for neutral lipids (solvent systems I-IV).

The mobility of free fatty acids on the new sintered plate (unwashed sintered plate) differed from that on the washed sintered plate (Rf value of free fatty acid 0.14 for former and 0.43 for latter), some polar lipids showing a similar tendency.

Since two dimensional TLC is an essential tool for the complete resolution of complex polar lipid mixtures found in nature, one of the purposes of this experiment was to see if the sintered plate could be used for two dimensional TLC. Figure 2 shows the separation of a complex mixture (ca. 70 μg) of total lipid extracts from yeast cells *(Tricosporone cutanium KC 4-3)*, supplemented by LPC, SPM, PG and CMH. Combined developing systems VI and VII on washed sintered plates could clearly separate most of the naturally occurring complex phospholipids. X_1, X_2, X_3 and X_4 in Figure 2 were the unidentified lipids found in the yeast lipids extract (to be discussed in a later paper). The washed sintered plate gave better resolution of complex polar lipids mixtures for all combinations of solvent systems listed in Materials and Methods. However the mobilities of some acidic phospholipids such as PS, PI, PA and CL on unwashed sintered plates were different from their mobilities on washed sintered plates in solvent systems VI, VIII and IX. Various sodium salts present in the silica gel adsorbent greatly affect the mobility of some phospholipids in TLC using silica gel coatings (14). The same phenomenon was observed on

TABLE I

Rf Values of Neutral Lipids on Washed Sintered Plates

Substances	Solvent systems					
	I	I[a]	II	IV	III	V
1-Monostearin	Origin	Origin	0.15			
Octadecyl glyceryl ether	Origin	Origin	0.16			
1,2-Dipalmitin	0.07	0.10	0.61			
1,3-Dipalmitin	0.11	0.14	0.68			
Cholesterol	0.15	0.14	0.57			
Cetyl alcohol	0.22	0.18	0.65			
Stearic acid	0.43	0.14	0.82			
Triglycerides (prepared from olive oil)	0.52	0.47	0.89	0.09		
Diacyl glyceryl ether	0.67	0.62		0.09		
Fatty acid methyl esters (prepared from egg yolk phospholipids)	0.75	0.77		0.40		
Palmitoyl cholesterol	0.90	0.89		0.73	0.02	
Squalene				0.83	0.37	
Squalane	0.93	0.93		0.88	0.82	
Bee wax	0.93	0.93		0.92	0.84	
Vitamin E						0.56
Vitamin A acetate						0.72
Vitamin K_1						0.84
Vitamin A palmitate						0.91

[a]On unwashed sintered plates.

unwashed sintered plates and sintered plates impregnated with sodium bicarbonate. Therefore the silica gel on new sintered plates probably contains various sodium salts derived from the raw material adsorbent. This would explain the difference in the mobilities of acidic phospholipids and fatty acids between washed and new plates, if the chromic-sulfuric acid cleaning solution could remove the sodium salts. By utilizing the characteristics of unwashed sintered plates and sintered plates impregnated with sodium bicarbonate (Fig. 3), we have separated CL and an unidentified phospholipid found in lipid extracts from yeast cells *(Cry. neoformans CBS-132)* (8).

Since silica gel-coated plates are fragile, it is necessary to spray the chromatogram with polyvinyl alcohol (13) before performing the liquid phase reaction. The sintered plate was very sturdy and could be immersed without special precautions in a number of liquid reaction mixtures, such as oil red staining solution (9), Sudan black staining solution (10) and Levine-Chargaff reagent (13). Because it is probable that the lipids on the chromatogram would react more uniformly with the reagent in the liquid phase than by spraying methods, this procedure may be applicable to quantitative densitometry.

Argentation-sintered plates were easily prepared by immersing the washed sintered plates in 12.5% silver nitrate solution and activating at 120 C for 2 hr. Figure 4 is a chromatogram of some fatty acid methyl esters on the argentation-sintered plate.

The sintered plate proved useful for reversed phase partition TLC (Fig. 5).

The chromatographic qualities of the sintered plates did not deteriorate after they were used 20 times, and we think they will stand up to many periods of continuous use and are therefore very economical.

The sintered plate is therefore a significant development for silica gel TLC in electrophoresis and quantitative analysis, except for preparative TLC.

REFERENCES

1. Kaneko, H., and Y. Kawanishi, Yukagaku 12:597 (1963).
2. "Methods in Enzymology," Vol. XIV, Edited by J.M. Lowenstein, Academic Press, New York, 1969, p. 530.
3. "Lipid Chromatographic Analysis," Vol. I, Edited by G.V. Marinetti, Marcel Dekker, Inc., New York, 1967.
4. Okumura, T., and T. Kadono, Japan Analyst 21:321 (1972).
5. Palameta, B., and M. Kates, Biochemistry 5:618 (1966).
6. Newman, H.A.I., C. Liu and D.B. Zilversmit, J. Lipid Res. 2:403 (1961).
7. Chang, T.L., and C.C. Sweeley, Biochemistry 2:592 (1963).
8. Itoh, T., and H. Kaneko, Presented at the Japan Agricultural Chemists' Society Meeting, Tokyo, April 1971, Abstr. 21-07.
9. Uriel, J., and P. Graber, Bull. Soc. Chim. Biol. 38:1253 (1956).

10. Uriel, J., P. Graber and C. Wunderly, Clin. Chim. Acta 2:35 (1957).
11. Dittmer, J.C., and R.L. Lester, J. Lipid Res. 5:126 (1964).
12. Skipski, V.P., A.F. Smolowe and M. Barclay, Ibid. 8:295 (1967).
13. Schneider, P.B., Ibid. 7:169 (1966).
14. Itoh, T., and H. Kaneko, Yukagaku 20:23 (1971).

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Lipoxygenase in *Chlorella pyrenoidosa*

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ABSTRACT

The presence of lipoxygenase enzyme is observed when cells of *Chlorella pyrenoidosa* are homogenized under anaerobic conditions. This is the first report of this enzyme in a lower form of plant life. The major product of *Chlorella* lipoxygenase with linoleic acid as substrate is 13-hydroperoxyoctadecadienoic acid.

INTRODUCTION

The enzyme lipoxygenase (EC 1.13.1.13) catalyzes the oxidation of linoleic acid and certain other polyunsaturated fatty acids, to form conjugated diene hydroperoxides, but its physiological function in plants remains a mystery. Early investigations indicated its presence in a number of leguminous and cereal seeds, and the enzyme from soybeans was extensively investigated (1). To the best of our knowledge, there has been no published report of lipoxygenase activity in algae. Our initial experiments to demonstrate the presence of lipoxygenase in green algae were unsuccessful. However, when care was taken to exclude oxygen from *Chlorella pyrenoidosa* cells during homogenization, activity could be observed with the homogenate.

MATERIALS AND METHODS

Chlorella pyrenoidosa (strain 211-8B) were grown in the light (10,000 lux) at 25 C in a modified Arnon's medium (2) and 1600 ml of cells (2.91 x 10^6 cells/ml) harvested during the log phase of growth. The cells were homogenized with 45 ml of glass beads (0.28 mm diameter) in 30 ml of deoyxgenated medium consisting of 0.2 M sucrose, 0.01 M NaCl and 0.05 M sodium phosphate buffer, pH 7.4. The supernatant, after centrifugation at 1000 x g for 10 min, was assayed directly or treated with saturated ammonium sulfate solution to make it 42% saturated. After centrifugation the precipitate was suspended in 2 ml 0.05 M sodium phosphate buffer, pH 7.4 and added to 120 ml phosphate buffer containing linoleic or linolenic acid (2.45 x 10^{-4} M) emulsified with 0.25% Tween 20 (3). After 1 hr, 30 min at 25 C, the mixture was acidified, extracted with petroleum ether, and assayed for lipoxygenase reaction products by thin layer chromatography

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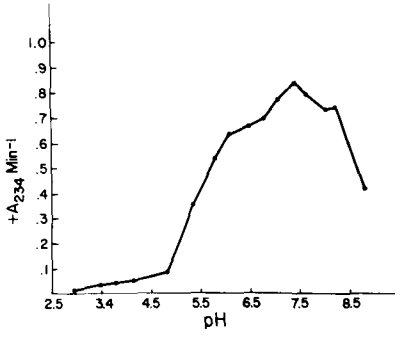

FIG. 1. Activity curve for *Chlorella* lipoxygenase at different pHs. Rates were determined in 0.05 M buffers: pH 2.9-5.5, sodium citrate; pH 5.8-8.0, sodium phosphate; pH 8.9, sodium borate.

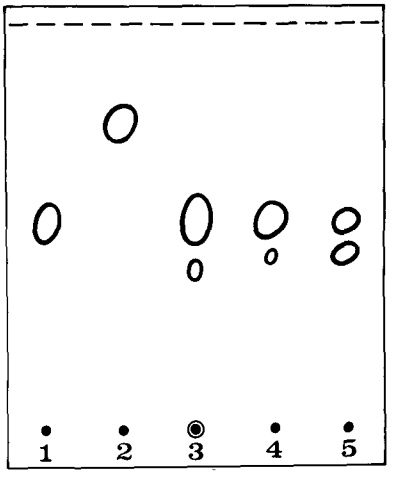

FIG. 2. Thin layer chromatogram of the methyl ester reaction products of *Chlorella* lipoxygenase. 1. Linoleic acid hydroperoxide resulting from commercial soybean lipoxygenase (Sigma Chemical Co.). 2. Linoleic acid plus heat-inactivated *Chlorella* fraction (42% saturated ammonium sulfate precipitate). 3. Linoleic acid hydroperoxides resulting from incubation of linoleic acid with *Chlorella* fraction. 4. Monohydroxy stearates produced by reduction of hydroperoxides in 3. 5. Methyl 12-hydroxystearate (upper spot) and methyl 9-hydroxystearate. Adsorbosil-5-silica gel with petroleum ether-diethyl ether-acetic acid 60:40:1 v/v solvent. Hydroperoxides were visualized by spraying with 0.1% N,N-dimethyl-*p*-phenylenediamine in chloroform-acetic acid-water 5:5:1 v/v. Other compounds were located by charring after spraying with a 70% sulfuric acid solution saturated with potassium dichromate.

(TLC). Reduction of hydroperoxides by sodium borohydride was followed by hydrogenation with palladium on charcoal, esterification with 14% BF_3 in methanol and purification by TLC, as described previously (3). Mass spectrographs were obtained with a Varian M66 mass spectrometer (3). Lipoxygenase activity was determined by both the spectrophotometric method for conjugated diene (4) and by the colorimetric peroxide determination (5).

RESULTS

When extracts of Chlorella cells were prepared under aerobic conditions, there was no evidence of lipoxygenase activity. By homogenizing the cells under nitrogen in a deoxygenated medium, lipoxygenase activity was readily observed. Incubation of a crude extract with linoleic acid showed an increase in absorbance at 234 nm, followed by a decrease. Colorimetric peroxide determinations also revealed an increase followed by a decrease, indicating that the formation and loss of conjugated diene is accompanied by a formation and loss of hydroperoxide. The loss of conjugated diene and hydroperoxide is believed due to the presence of a hydroperoxide isomerase (4), but positive identification of the reaction products has not been completed. Ammonium sulfate fractionation of the 1000 x g supernatant yielded a fraction (42% saturated) that exhibited only lipoxygenase activity. This fraction was used for the data reported here.

Chlorella lipoxygenase was active with both linoleic and linolenic acids, the latter ca. 30% more active than the former, but inactive with oleic acid. The variation of activity with pH revealed an optimum at 7.4 (Fig. 1). There was a trace of activity at pH 2, but none at pH 12, ruling out the possibility that the oxidation might be heme-catalyzed (6). In addition, 1.6 x 10^{-3} M sodium cyanide did not inhibit the activity. Activity was destroyed by heating the extract for 2 min at 100 C. Nordihydroguairetic acid, 2 x 10^{-4} M, inhibited the lipoxygenase 100%. Addition of 5 x 10^{-4} M calcium chloride did not affect lipoxygenase activity. Lipoxygenase activity of the crude extract was +2.5 A_{234}/min mg fresh weight of algal cells with linoleic acid substrate. This is the same order of magnitude as flaxseed lipoxygenase.

The products formed on incubation of the *Chlorella* 42% ammonium sulfate fraction with linoleic acid were isolated and analyzed by TLC. The results (Fig. 2) show that this fraction converts linoleic acid to hydroperoxide derivatives. When the hydroperoxides were reduced to their corresponding hydroxystearates and spotted on a thin layer plate, the R_fs corresponded to those of monohydroxy derivatives.

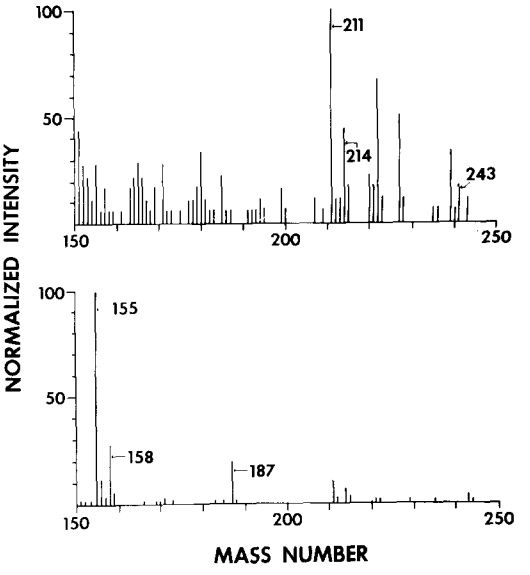

FIG. 3. Top: partial mass spectrum of major product of linoleic acid oxidation by *Chlorella* lipoxygenase, after reduction (Fig. 2, column 4, upper spot). Bottom: partial mass spectrum of minor product of linoleic acid oxidation by *Chlorella* lipoxygenase, after reduction (Fig. 2, column 4, lower spot).

Mass spectrometry of the major monohydroxy derivative isolated by TLC revealed mass peaks 211, 214 and 243 of methyl 13-hydroxystearate (Fig. 3)(3). The origin of mass peaks 222, 227 and 239 is not clear, although they have been observed by others in mass spectra of hydroxy methyl stearates (7). Dehydration in the fatty acid chain followed by loss of a (CH_2-CO-OCH_3 + H^+) fragment can account for their appearance. Mass peaks 155, 158 and 187 of methyl 9-hydroxystearate were obtained from the monohydroxy derivative immediately below the major compound (Fig. 2). The products of *Chlorella* lipoxygenase activity with linoleic acid appear to consist of ca. 80% 13-hydroperoxyoctadecadienoic acid and 20% 9-hydroperoxyoctadecadienoic acid, based on the separation obtained on thin layer plates (Fig. 2).

The function of lipoxygenase in plants is still undetermined. Although several possibilities have been suggested, none have been positively defined. The variety of products obtained with soybean lipoxygenase under anaerobic conditions in vitro has recently been reported (8), and the availability of molecular oxygen in vivo is a major consideration in assigning a physio-

logical role for the enzyme. Hopefully this report of lipoxygenase in the green alga *Chlorella pyrenoidosa* will provide a stimulus for further research by others in this area.

ACKNOWLEDGMENTS

M.E. Duysen provided the *Chlorella* cells. This paper was published with the approval of the director, North Dakota Agricultural Experiment Station, as Journal Article No. 390.

REFERENCES

1. Holman, R.T., and S. Bergstrom, in "The Enzymes," Vol. 2, Edited by J.B. Sumner and K. Myrback, Academic Press, New York, 1951, p. 559.
2. Budd, T.W., J.L. Tjostem and M.E. Duysen, Amer. J. Bot. 56:540 (1969).
3. Zimmerman, D.C., and B.A. Vick, Lipids 5:392 (1972).
4. Zimmerman, D.C., and B.A. Vick, Plant Physiol. 46:445 (1970).
5. Koch, R.B., Arch. Biochem. Biophys 78:165 (1958).
6. Siddiqi, A.M., Acta Biol. Acad. Sci. Hung. 22:275 (1971).
7. Ryhage, R., and E. Stenhagen, Arkiv Kemi 15:545 (1960).
8. Garssen, G.J., J.F.G. Vliegenthart and J. Boldingh, Biochem. J. 122:327 (1971).

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Mass Spectra of Prostaglandins: II. Trimethylsilyl and Alkyloxime-Trimethylsilyl Derivatives of Prostaglandins B_1 and B_2[1]

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ABSTRACT

The mass spectra of the trimethylsilyl ester trimethylsilyl ether derivatives of prostaglandins B_1 and B_2, and of their O-ethyl and O-methyl oximes are reported and discussed. The high resolution spectra of these compounds are also considered. These spectra are compared with those of the corresponding d_9-trimethylsilyl derivatives and of the selectively labeled trimethylsilyl ester d_9-trimethylsilyl ethers. The base peaks of the spectra of the trimethylsilyl derivatives are formed by sequential loss of C_5H_{11} (by cleavage of C-15/6) and of CO (from the ring). The spectra of the oxime-trimethylsilyl derivatives are dominated by ions resulting from loss of the alkoxy group of the oxime moiety.

INTRODUCTION

Prostaglandins of the E series are, by virtue of their 1,2-ketol moieties, thermally somewhat unstable. They are readily converted to the corresponding prostaglandins of the B series by treatment with bases (2-7) and, as such, are more amenable to vapor phase methods of analysis (8). In continuation of our studies (1) of the mass spectrometry (MS) of trimethylsilyl (TMS) derivatives of prostaglandins and their methyl oximes (MO) and ethyl oximes (EO), we now report on these derivatives of the B series. All elemental compositions were confirmed by high resolution data.

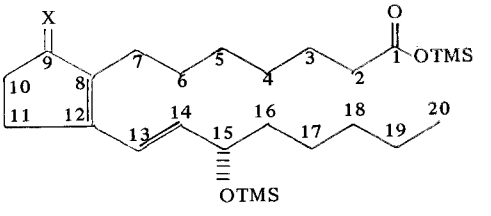
RESULTS AND DISCUSSION

The TMS derivatives of prostaglandins B_1 (I) and B_2 (II) give a single peak on gas chromatography (GC). In contrast to those of the A series, the MO-TMS (III, IV) and EO-TMS (V, VI) derivatives also afford a single peak. It is possible that *syn* and *anti* isomers have similar retention times, but it seems more likely that only one is formed, for stereochemical reasons. This is an important factor for quantitative analytical work, since it may lead to increased sensitivity.

Since the spectra of the TMS derivatives (Figs. 1-2) are strikingly different from those of the MO-TMS (Figs. 3-4) and EO-TMS (Figs. 5-6) derivatives, it is convenient to discuss each class of compound in turn.

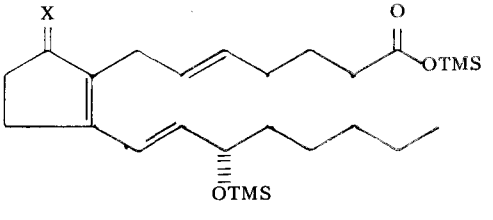
TMS Derivatives

The mass spectrum of PGA_1-TMS (I) is given in Figure 1, and the spectrum of PGA_2-TMS (II) in Figure 2. The molecular ions are of moderate intensity (I: m/e 480, 34%; II: m/e 478, 35%), and they can lose a methyl radical to afford relatively abundant $[M-15]^+$ ions (I: m/e 465, 22%; II: m/e 463, 30%). The presence of the 15-trimethylsilyloxy group promotes the loss of C_5H_{11} by cleavage of C-15/16 (1), leading to the formation of ions of m/e 409 (I, 29%) or m/e 407 (II, 20%). Elimination of trimethyl-



I: X = O
III: X = NOMe
V: X = NOEt

Structures of PGB_1 TMS derivatives.



II: X = O
IV: X = NOMe
VI: X = NOEt

Structures of PGB_2 TMS derivatives.

[1] For Part I, see Reference 1.

[2] Fellow of the Intra-Science Research Foundation (1971-75).

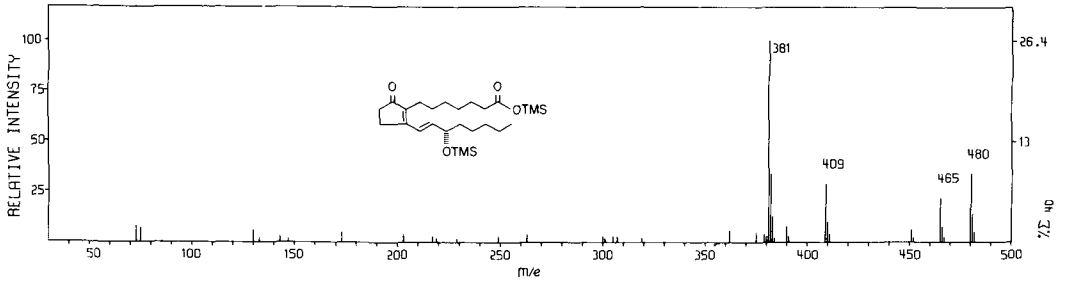


FIG. 1. Mass spectrum (22.5 eV) of TMS derivative of prostaglandin B_1 (I).

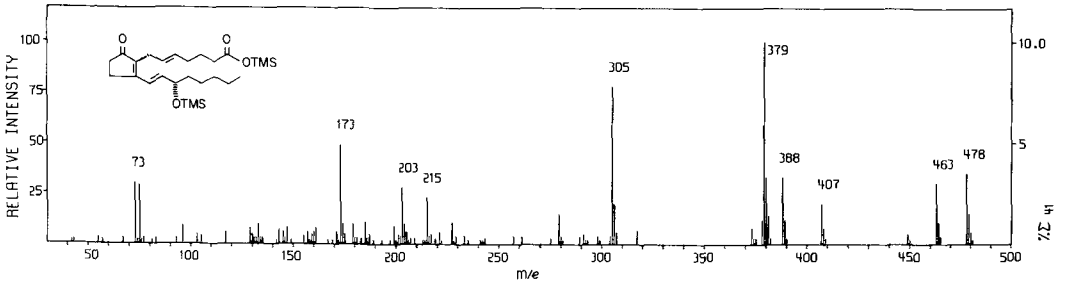


FIG. 2. Mass spectrum (22.5 eV) of TMS derivative of prostaglandin B_2 (II).

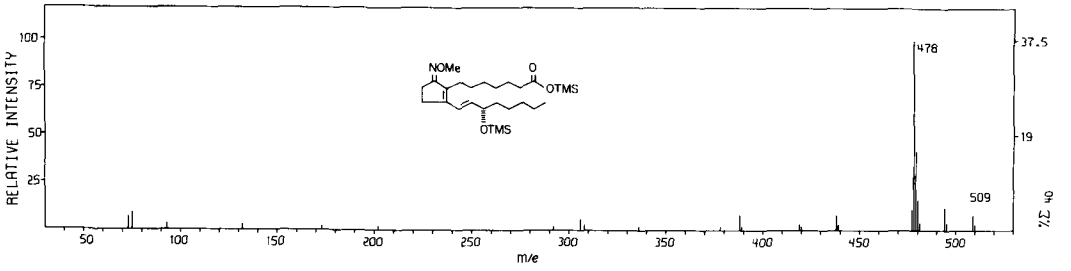


FIG. 3. Mass spectrum (22.5 eV) of MO-TMS derivative of prostaglandin B_1 (III).

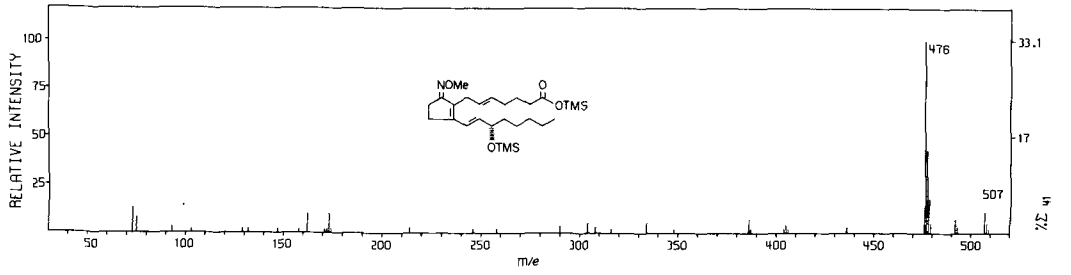


FIG. 4. Mass spectrum (22.5 eV) of MO-TMS derivative of prostaglandin B_2 (IV).

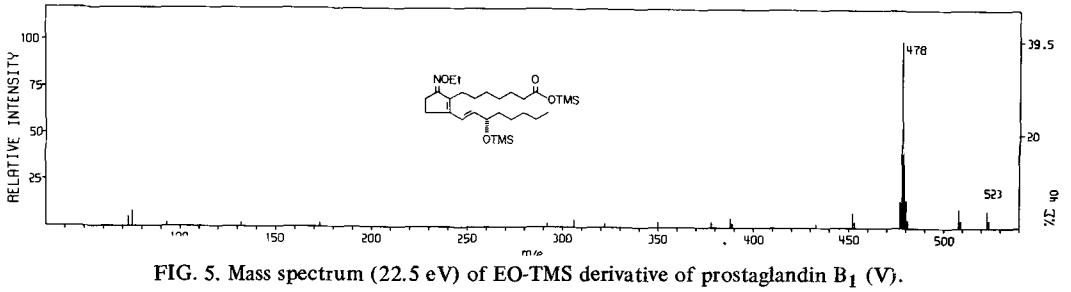


FIG. 5. Mass spectrum (22.5 eV) of EO-TMS derivative of prostaglandin B_1 (V).

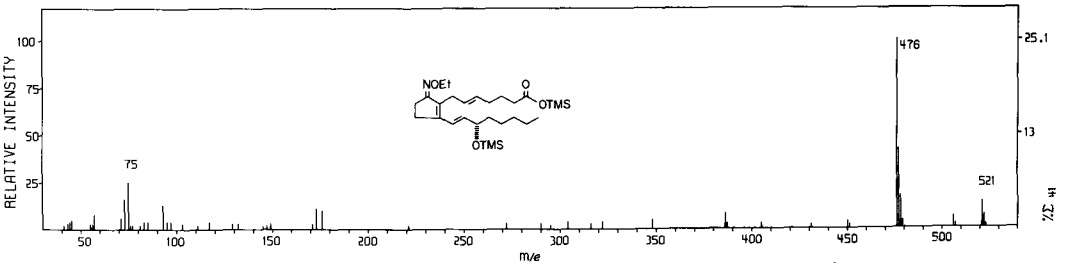


FIG. 6. Mass spectrum (22.5 eV) of EO-TMS derivative of prostaglandin B_2 (VI).

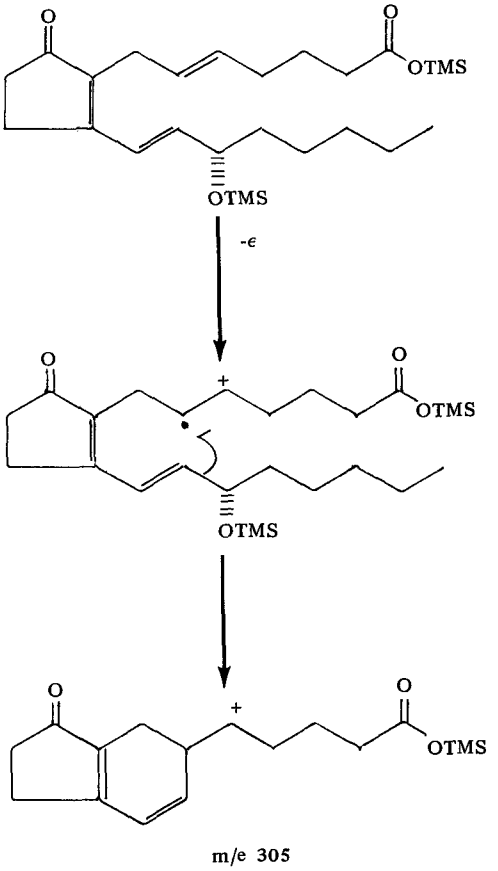
silanol from the molecular ions to give $[M-90]^+$ ions (I: m/e 390, 8%; II: m/e 388, 33%) and subsequent loss of a methyl radical to afford ions of m/e 375 (I, 5%) and m/e 373 (II, 9%) also take place. Elimination of trimethylsilanol from the $[M-71]^+$ ions provides ions of only low intensity (I: m/e 319, 2%; II: m/e 317, 7%).

Each of these ions is also present in the spectrum of the TMS derivative of prostaglandin A_1 (1). In the spectra of TMS derivatives of prostaglandins of the B series, there are certain additional characteristic fragmentations. The base peak of each spectrum is formed by elimination of CO from the $[M-71]^+$ ion (I: m/e 381, II: m/e 379). The analogous fragmentation of the corresponding derivative of prostaglandin A_1 gives rise to such an ion in much lower abundance (1), but it seems likely that similar mechanisms apply, the CO being lost from the ring carbonyl group. It should be noted that CO is also lost from the $[M-90]^+$ ion of I, to yield a relatively weak ion of m/e 362 (6%). Weak ions are also formed by loss of HCO from the molecular ions (I: m/e 451, 6%; II: m/e 449, 5%). It appears that these ions also involve loss of the ring carbonyl group, but with an additional hydrogen atom.

The fragmentations so far discussed account for the major ions in the spectrum of I, but additional intense ions are seen in the spectrum of II. The extremely abundant ion of m/e 305 (78%) is apparently formed by cleavage of C-14/15, with charge retention on the larger fragment. This ion can eliminate trimethylsilanol to yield the ion of m/e 215 (23%). The rationale for the cleavage of C-14/15 and the reason for the influence of the Δ^5-bond on this fragmentation mode are uncertain. A formal possibility for this fragmentation mode is shown in Scheme 1. Formation of the ion of m/e 173 (49%) by cleavage of C-14/15 is probably also aided by simultaneous production of a relatively stable neutral particle of 305 mass units.

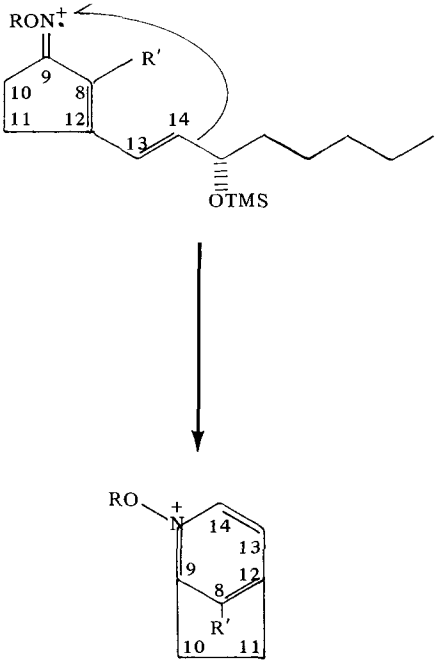
MO-TMS and EO-TMS Derivatives

The mass spectra of compounds III-VI are given in Figures 3-6, respectively. The base peaks in each of these spectra are formed by loss of the alkyloxy group of the oxime moiety from the molecular ion (m/e 478: III, 37.5%, V, 39.5%; m/e 476: IV, 33.1%, VI, 25.1% Σ_{40}). The large contributions of these ions to the total ion current (Σ_{40}), and the fact that only one of the *syn-anti* isomers is seen, indicate that MO-TMS derivatives should be considered for specific ion monitoring (8,9) of prostaglandins of the B series.



m/e 305

Scheme 1.



Scheme 2. From IIIa, IIIb: R = Me, R′ = $(CH_2)_6CO_2TMS$; m/e 336. From IVa, IVb: R = Me, R′ = $CH_2CH{=}CH(CH_2)_3CO_2TMS$; m/e 334. From Va, Vb: R = Et, R′ = $(CH_2)_6CO_2TMS$; m/e 350. From VIa, VIb: R = Et, R′ = $CH_2CH{=}CH(CH_2)_3CO_2TMS$; m/e 348.

Each of the other ions in the spectra is of low relative abundance when compared with the base peak, but several contribute 3-4% to the total ion current. The molecular ions (III: m/e 509, 8%; IV: m/e 507, 11%; V: m/e 523, 9%; VI: m/e 521, 14%) each lose a methyl radical from the ester TMS group to yield $[M-15]^+$ ions (III: m/e 494, 12%; IV: m/e 492, 7%; V: m/e 508, 10%; VI: m/e 506, 6%).

Also present in the spectra are the expected ions of the type $[M-71]^+$ (loss of C_5H_{11} by scission of C-15/16), $[M-90]^+$ (elimination of trimethylsilanol), and ions formed by successive loss of the alkoxy group of the oxime moiety and elimination of trimethylsilanol (m/e 388: III, 8%, V, 5%; m/e 386: IV, 7%, VI, 8%).

It was postulated that loss of 173 mass units (C-15/20, with substituents) from the molecular ion of II took place with participation of the Δ^5 bond, as shown in Scheme 1. There was no analogous fragmentation of I. The spectra of the MO-TMS and EO-TMS derivatives of prostaglandins of the B series all contain $[M-173]^+$ ions whether or not a Δ^5 bond is present: (III: m/e 336, 2%; IV: m/e 334, 5%; V: m/e 350, 3%; VI: m/e 348, 5%). The participation of the oxime group in the formation of these ions may be represented as in Scheme 2. It is possible, also, that at least part of the $[M-173]^+$ ions in the spectra of IV and VI is formed as in Scheme 1.

CONCLUSIONS

The spectra of prostaglandins of the B series are quite different from those of the A series, the two series differing only in the position of a double bond. Moreover, while the oxime-TMS derivatives of the former give only one peak on GC, the latter give two (10,11).

When the spectra of the TMS derivatives of prostaglandins of the A and B series are compared, it is found that the $[M-99]^+$ ions predominate in the spectra of the latter. It is also seen that the oxime-TMS derivatives of the A series fragment more randomly than those of the B series.

EXPERIMENTAL PROCEDURES

Prostaglandins were obtained from J.E. Pike and U. Axen (Upjohn Co., Kalamazoo, Mich.) and from K. Sano (Ono Pharmaceutical Co., Osaka, Japan).

Derivatives were prepared as previously described (1).

Mass spectrometry was performed using LKB 9000 (low resolution, GC-MS) and CEC 21-110B (high resolution, direct insertion probe) instruments as previously described (1).

ACKNOWLEDGMENTS

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REFERENCES

1. Middleditch, B.S., and D.M. Desiderio, In press, Prostaglandins 1973.
2. Bergstrom, S., R. Ryhage, B. Samuelsson and J. Sjövall, J. Biol. Chem. 238:3555 (1963).
3. Corey, E.J., N.H. Andersen, R.M. Carlson, J. Paust, E. Vedejs, I. Vlahas and R.E.K. Winter, J. Amer. Chem. Soc. 90:3245 (1968).
4. Andersen, N.H., J. Lipid Res. 10:320 (1969).
5. Polet, H., and L. Levine, Biochem. Biophys. Res. Commun. 45:1169 (1971).
6. Levine, L., R.M.G. Cernosek and H. VanVunakis, J. Biol. Chem. 246:6782 (1971).
7. Zusman, R.M., Prostaglandins 1:167 (1972).
8. Sweetman, B.J., J.C. Frölich and J.T. Watson, Presented at the Fifth International Congress on Pharmacology, San Francisco, 1972.
9. Brooks, C.J.W., and B.S. Middleditch, Clin. Chim. Acta 34:145 (1971).
10. Vane, F., and M.G. Horning, Anal. Letters 2:357 (1969).
11. Middleditch, B.S., and D.M. Desiderio, Prostaglandins 2:115 (1972).

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Dimorphotheca sinuata Lipoxygenase: Formation of 13-L-Hydroperoxy-*cis*-9,*trans*-11-Octadecadienoic Acid from Linoleic Acid

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ABSTRACT

Lipoxygenase (EC 1.13.1.13) from the seed of *Dimorphotheca sinuata* oxidized linoleic acid to predominantly 13-L-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid. When the reaction proceeded at pH 6.9, the 13-hydroperoxide was the only isomer detected; but at pH 5.1, the 13-isomer was 92% of the total, the remaining 8% being the 9-hydroperoxide. At both pH's small amounts of hydroxyoctadecadienoic acid accumulated during the reaction. This acid from the pH 6.9 reaction was analyzed as 13-hydroxy-*cis*,*trans*-octadecadienoic. The postulate advanced by many workers that dimorphecolic acid, 9-D-hydroxy-*trans*-10,*trans*-12-octadecadienoic acid, is biosynthesized via a lipoxygenase product was not proved. Although the product specificity of *D. sinuata* lipoxygenase is like that of lipoxygenase type 1 from soybeans, its inactivity at pH 9 demonstrated that it is a novel enzyme.

INTRODUCTION

Lipoxygenase (EC 1.13.1.13) from *Dimorphotheca sinuata*, formerly known as *D. aurantiaca* (1), has not been reported previously.

Our research with *Dimorphotheca* was prompted to evaluate the postulate that dimorphecolic acid, the predominant fatty acid in the glycerides of the seed oil (1), is derived from a lipoxygenase product. Others (1-3) have already speculated on the possible involvement of lipoxygenase in the biosynthesis of dimorphecolic acid, 9-D-hydroxy-*trans*-10,*trans*-12-octadecadienoic acid. Both corn (3) and potatoes (4) contain lipoxygenases that oxidize linoleic acid to a potential dimorphecolic acid precursor, 9-D-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid. Hydroperoxide can be converted to hydroxyl with reducing agents of low potential, such as have been proposed for lipoxygenase in wheat dough through oxidation of SH-groups (5). Isomerization of the *cis,trans* double bond to the more thermodynamically stable *trans,trans* configuration occurs under acidic conditions but is accompanied by positional isomerization of the hydroxyl (6). That lipoxygenase may also be capable of producing a *trans,trans* isomer was suggested by the presence of 5% *trans,trans* isomer in the hydroperoxides derived from corn lipoxygenase (3,7). Whether this compound was an artifact or an actual enzyme product is open to speculation.

In working with *D. sinuata* lipoxygenase we found, instead of 9-D-specificity, that the oxidation was at the 13-L-carbon. This oxidation specificity is like that of the predominant soybean lipoxygenase activity which yields 13-L-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid from linoleic acid (8,9). According to Christopher et al. (10,11), there are three lipoxygenase isoenzymes in soybeans of which at least one, lipoxygenase type 1, is known to produce only the 13-hydroperoxide (12). The type 1 enzyme has its optimum activity at pH 9.5 (10).

We report here a lipoxygenase activity that is like soybean lipoxygenase type 1 in product specificity, but dissimilar in pH optimum.

METHODS

Lipoxygenase Assay

A water extract was prepared by homogeniz-

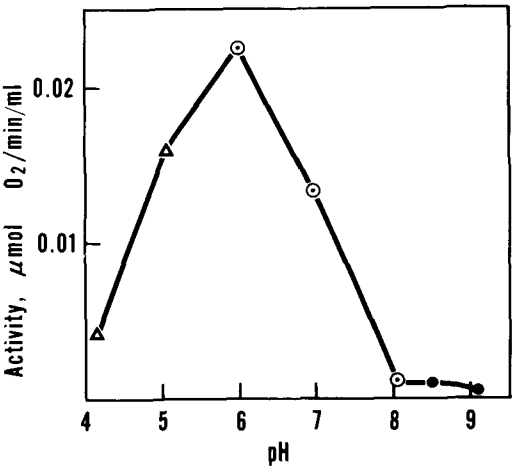


FIG. 1. Effect of pH on *Dimorphotheca sinuata* lipoxygenase activity. Buffers: ▲, acetate; ○, phosphate; ●, ammonium.

[1]ARS, USDA.

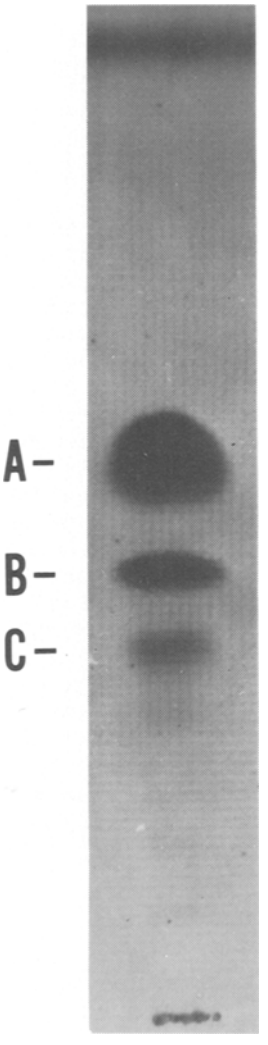


FIG. 2. Thin layer chromatogram of linoleic acid oxidation products. Solvent: isooctane-ether-acetic acid 50:50:1. A, unreacted linoleic acid; B, hydroperoxyoctadecadienoic acid; C, hydroxyoctadecadienoic acid. B was positive to ferrous thiocyanate spray.

ing hexane-defatted *D. sinuata* seed meal in the proportion of 7 g/100 ml. After centrifugation at 8000 g for 10 min, the resultant supernatant was fractionated with $(NH_4)_2SO_4$ at 0 C between 33 and 64% of saturation (13). The reaction mixture used to measure activity was 0.28% Tween-20, 8.4 mM linoleic acid (introduced into the reaction vessel as the K salt), 0.096 M buffer, and a quantity of $(NH_4)_2SO_4$ fraction equivalent to 33 mg seed meal per milliliter. The buffers employed were Na acetate, acetic acid; KH_2PO_4, Na_2HPO_4; and NH_4OH, NH_4Cl.

Activity was measured at 25 C by O_2 uptake

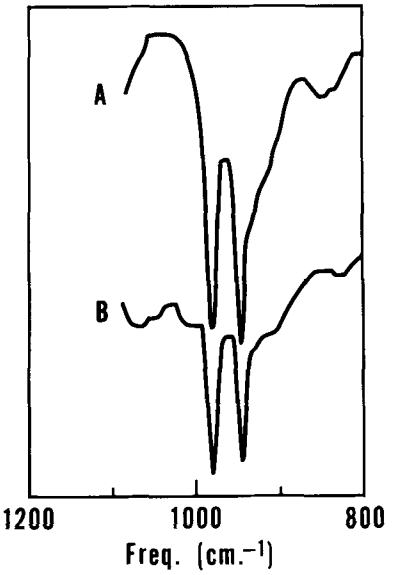


FIG. 3. IR absorption of *cis,trans* double bonds of hydroperoxyoctadecadienoic acid (A) and hydroxyoctadecadienoic acid (B), which were isolated from lipoxygenase product mixture. A, 10% in CS_2; B, 6% in CS_2 using 0.1 mm NaCl cell.

using an O_2 electrode apparatus, Gilson Oxygraph K-1C. The rate of O_2 uptake in the appropriate endogenous control was subtracted from the maximum rate observed with linoleic acid added.

Protein was determined by the method of Lowry et al. (14).

Electrophoresis

Lipoxygenase was separated on polyacrylamide gel by the disc electrophoretic method (15), with the exception that the separating gel was 5.25% polyacrylamide modified to contain starch for iodometry (16). Before electrophoresis, the following aqueous extracts were prepared: 2 g *D. sinuata* meal, 1 g soybeans and 180 mg lyophilized corn germ extract per 6 ml water. After centrifugation, the following quantities of supernatant were mixed with an equal volume of 40% sucrose: 30 µl *D. sinuata*, 7 µl soy (diluted five-fold with water) and 20 µl corn. The samples were then layered over large-pore stacking gels. All other conditions were the same as reported by Guss et al. (16).

After electrophoresis, the gels were immersed in a K linoleate (32 mM)-1% Tween-20 solution at pH 9.2 for 30 min at room temperature to impregnate the gels with linoleate and permit any lipoxygenase with a pH 9 optimum to react. Next the gels were submerged in 0.2 M phosphate buffer (pH 6.4) for 30 min to ensure that isoenzymes having a

lower pH optimum would react. After incubation the gels were stained with KI according to the method of Guss et al. (16).

Product Formation

An $(NH_4)_2SO_4$ fraction of a seed meal extract was prepared as described in the Lipoxygenase Assay Section and then taken up in a volume of either acetate or phosphate buffer. The buffered extract was transferred to a vessel of sufficient size to ensure good surface exposure, and the vessel was flushed with pure oxygen. A volume of K linoleate-Tween-20 solution was added with vigorous stirring which continued during the reaction time of 34 min at 23 C. The final reaction mixture was 7.7 mM linoleic acid, 0.044% Tween-20, 0.1 M buffer and equivalent to 72 mg of the original seed meal per milliliter.

The reaction was terminated by extraction of the fatty acids with triple the volume of chloroform-methanol 2:1.

Derivatives

Derivatives were synthesized by methods reported earlier (3). In certain experiments the hydroperoxyoctadecadienoic acid was hydrogenated directly to hydroxystearic acid in methanol with a 10% palladium catalyst on charcoal. Veldink (7) found that hydrogenation without prior use of agents to reduce the hydroperoxide was more reliable in retaining the original position of the oxygenated carbon.

Trimethylsiloxy (TMS) derivatives of methyl hydroxystearates and hydroxyoctadecadienoic acid were prepared (17).

Chromatography

Thin layer chromatography (TLC) on Silica Gel G plates either 250 μ or 1 mm thick was used to isolate products. The solvent employed to separate nonesterified compounds was redistilled hexane-ether-acetic acid 60:40:1. TLC scrapings of isolated products were slurried with chloroform-methanol 2:1 and filtered through a few centimeters of *Mallinckrodt* 100 mesh silicic acid.

The ratio of methyl 9- and 13-hydroxystearates in mixtures was determined by TLC densitometry (3).

Spectroscopy

Optical rotation was measured by a Bendix Model 1169 automatic polarimeter at 546 nm in a 0.2 dm cell. Gas chromatography-mass spectrometry (GC-MS) was applied as described by Kleiman and Spencer (17). The GC peaks were sampled five times during elution to obtain mass spectra representative of the entire peak. All other spectra were determined as

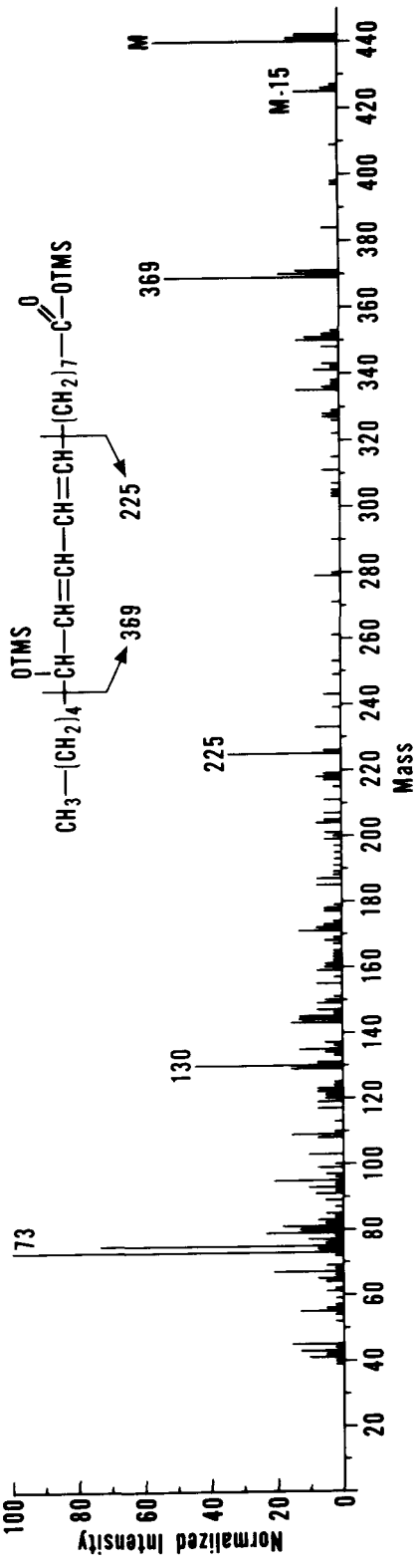
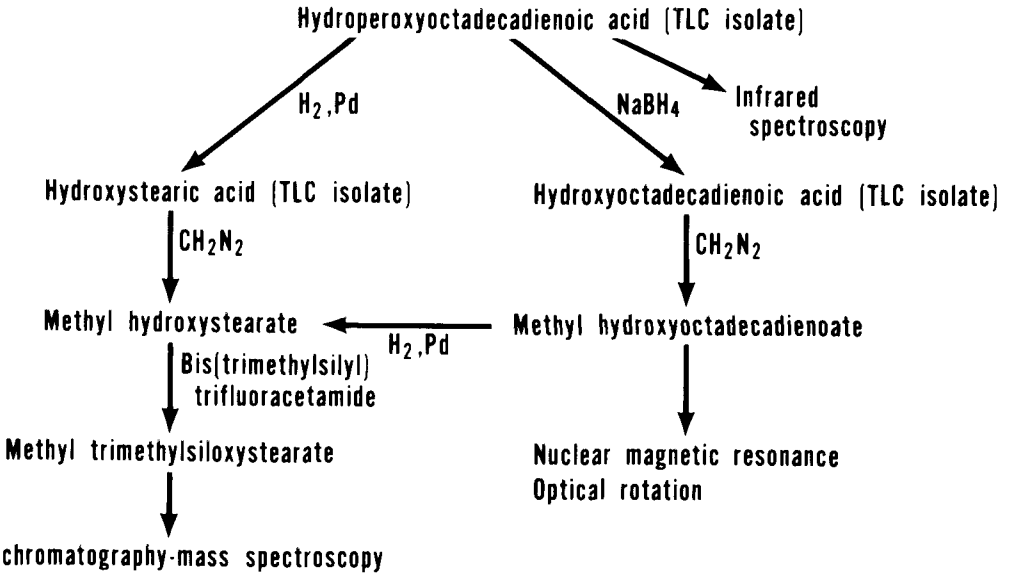


FIG. 4. Mass spectrum of trimethylsilyl trimethylsiloxyoctadecadienoate derivatized from hydroxyoctadecadienoic acid; minor product formed from linoleic acid in *D. sinuata* extracts.



Scheme 1. Derivatives from hydroperoxyoctadecadienoic acid.

previously described (3).

RESULTS

Effect of pH

The pH profile of *D. sinuata* lipoxygenase (Fig. 1) indicates an optimum at pH 6 and virtually no activity at pH 8-9.

Specific Activity

Apparently the lipoxygenase activity in extracts we prepared from the sample of *D. sinuata* seed was low compared to activities from other plant species. The highest activity observed at pH 6.9 with the $(NH_4)_2SO_4$ isolate was 3.9 x 10^{-3} units (μmol O_2/min)/mg protein. By comparison, our unpublished results with crude corn germ extracts at pH 6.9 yielded specific activities much higher, 0.10-0.75 units per mg protein. Other plant materials have even greater lipoxygenase concentrations, such as potato (1.2 units per mg protein) (4) and soybeans (1.37 units per mg dry matter) (18).

Oxidation Products

When linoleic acid was incubated with *D. sinuata* lipoxygenase, two detectable acids were formed, hydroperoxyoctadecadienoic and hydroxyoctadecadienoic. These acids were separated by TLC (Fig. 2).

Hydroperoxyoctadecadienoic acid: When the hydroperoxyoctadecadienoic acid, isolated in 15% yield from a lipoxygenase reaction at pH 6.9, was analyzed by IR spectroscopy (IR), a *cis,trans* conjugated diene was revealed (Fig. 3A). The isolate was further characterized after it was derivatized according to Scheme 1. A NMR spectrum of the methyl hydroxyoctadecadienoate derivative was identical to a spectrum of methyl coriolate (19); this similarity indicated the geometry of the dienol was α,β-*trans*-γ,δ-*cis*. The optical rotation of the methyl hydroxyoctadecadienoate derivative was $[\alpha]^{23.5}_{546} = +7.0°$ (C, 0.84% hexane), which is the same direction and approximate magnitude of rotation reported for methyl L-coriolate (2). To discover the position of the oxygenated carbon, the hydroperoxyoctadecadienoic acid was hydrogenated either directly or after reduction with $NaBH_4$ (Scheme 1). The methyl hydroxystearate prepared by either method was discovered to be oxygenated 100% at the 13-carbon as determined by GC-MS and TLC.

Products were isolated from an oxidation mixture reacted at pH 5.1 to test the acidic side of the pH curve for specificity of the oxidation. A TLC-densitometry analysis of the fully derivatized hydroperoxyoctadecadienoic acid (Scheme 1) showed that carbon-13 was oxygenated 92 ± 0.5%, a percentage almost identical to the results obtained on the pH 6.9 side of the optimum. The remaining 8% was oxygenated at carbon-9. GC-MS confirmed TLC results but was not quantitated.

Hydroxyoctadecadienoic acid: Compared to the hydroperoxide, hydroxyoctadecadienoic acid was a minor product of the lipoxygenase-catalyzed oxidation. A TLC isolate was determined to be a conjugated *cis,trans*-dienol by IR (Fig. 3B). The TMS derivative of the isolate, trimethylsilyl trimethylsiloxyoctadecadi-

enoate, was analyzed by GC-MS (Fig. 4). The position of the hydroxyl was determined by GC-MS after hydrogenation, as was done with the hydroperoxide. The 13-hydroxyl was the only isomer detected. Thus this product was 13-hydroxy-*cis,trans*-octadeca-9,11-dienoic acid and, presumably, would have geometry like the hydroperoxide, i.e., *cis*-9,*trans*-11.

Electrophoresis

A water extract of *D. sinuata* seed meal was applied to 5.25% polyacrylamide gel for electrophoresis (Fig. 5). The mobility of *D. sinuata* lipoxygenase compared with that of corn lipoxygenase, a predominantly 9-oxygenating lipoxygenase, and with the most mobile of the soybean isoenzymes, lipoxygenase type 1 (10). In our hands, the specific stain for lipoxygenase failed to develop in a high percentage of trials with corn and *D. sinuata* lipoxygenases. The reason for this failure is not apparent but may be due to a requirement for a threshold amount of hydroperoxide, which must be achieved to obtain release of I_2 in the gel. By limiting the amount of soybean extract applied to the gel, we have been able to reduce several isoenzyme bands to only one.

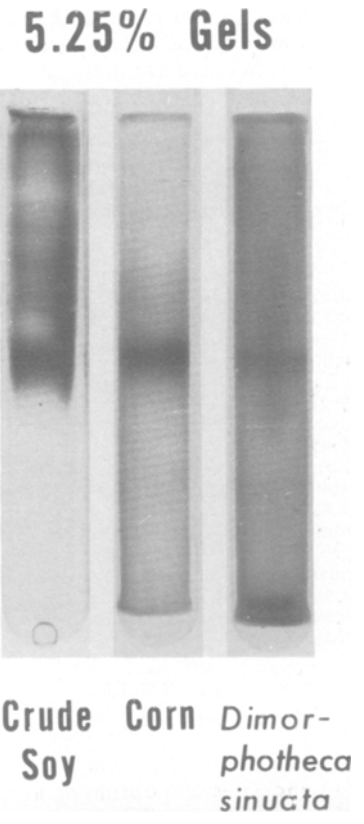


FIG. 5. Polyacrylamide gel electrophoresis of lipoxygenases from various sources. Bands were specifically stained for enzyme.

DISCUSSION

As a result of our experimentation with *D. sinuata*, we concluded that lipoxygenase may not be responsible for the biosynthesis of dimorphecolic acid, which makes up two-thirds of the fatty acids in the seed oil. This conclusion contradicts the hypothesis of numerous workers (1,3,7,20). However the possibility remains that a different lipoxygenase activity exists in the formative stage of seed ripening when the fatty acids are being biosynthesized.

A unique feature of *D. sinuata* lipoxygenase activity is the production of the 13-L-isomer in high yield at low pH values. In oxidation specificity, it is like soybean lipoxygenase type 1 (12). Conversely, the *D. sinuata* enzyme has a pH curve more like lipoxygenases specific for oxidation at the 9-D-carbon, such as corn (3) or potato (4), or like the enzyme specific for both 9- and 13-oxidation, such as lipoxygenase type 2 from soybean (10,12). From the evidence, it appears that the lipoxygenase activity in *D. sinuata* has no other precedent in the literature. Although many lipoxygenase activities from different sources are similar in positional specificity, another variable, the pH curve when available, defines each activity in the literature as unique to date.

That the 13-isomer was 100% of the product at pH 6.9 and 92% at pH 5.1 indicated a general lack of specificity dependence on pH. A pH dependency has been reported for *Zea mays* (21) and soybean lipoxygenase (22). With partially purified soy lipoxygenase, apparently specificity dependency on pH is due to the influence of at least two isoenzymes that have different pH optima. The same may be true of *Z. mays* lipoxygenase, but the existence of isoenzymes has not been investigated. We tested both sides of the pH optimum of *D. sinuata* lipoxygenase for positional specificity to allow for interaction of isoenzymes; however one isomer predominated. The polyacrylamide gel also indicates the existence of one lipoxygenase. Due to a possible threshold phenomenon in staining the gels, it is not certain whether some isoenzymes remain undetected.

The hydroxyoctadecadienoic acid in the lipoxygenase oxidation mixture undoubtedly was formed from the hydroperoxide since its structure was similar. Extracts of oat (23) and wheat flour (5) have been observed to reduce linoleic acid hydroperoxide to the corresponding hydroxyoctadecadienoic acids. Other than

the conversion to hydroxyoctadecadienoic acid, no other significant secondary reaction seemed to occur even in crude extracts. The presence of linoleic acid hydroperoxide isomerase was *not* obvious.

ACKNOWLEDGMENT

D. Weisleder supplied the NMR spectrum.

REFERENCES

1. Smith, C.R., T.L. Wilson, E.H. Melvin and I.A. Wolff, J. Amer. Chem. Soc. 82:1417 (1960).
2. Phillips, B.E., C.R. Smith, Jr., and L.W. Tjarks, Biochim. Biophys. Acta 210:353 (1970).
3. Gardner, H.W., and D. Weisleder, Lipids 5:678 (1970).
4. Galliard, T., and D.R. Phillips, Biochem. J. 124:431 (1971).
5. Graveland, A., JAOCS 47:352 (1970).
6. Powell, R.G., C.R. Smith, Jr., and I.A. Wolff, J. Org. Chem. 32:1442 (1967).
7. Veldink, G.A., Ph.D. thesis, University of Utrecht, 1971.
8. Hamberg, M., and B. Samuelsson, J. Biol. Chem. 242:5329 (1967).
9. Gardner, H.W., and D. Weisleder, Lipids 7:191 (1972).
10. Christopher, J.P., E.K. Pistorius and B. Axelrod, Biochim. Biophys. Acta 198:12 (1970).
11. Christopher, J.P., E.K. Pistorius and B. Axelrod, Ibid. 284:54 (1972).
12. Christopher, J.P., and B. Axelrod, Biochem. Biophys. Res. Commun. 44:731 (1971).
13. Jeso, F. di, J. Biol. Chem. 243:2022 (1968).
14. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, Ibid. 193:265 (1951).
15. Davis, B.J., Ann. N.Y. Acad. Sci. 121:404 (1964).
16. Guss, P.L., T. Richardson and M.A. Stahmann, Cereal Chem. 44:607 (1967).
17. Kleiman, R., and G.F. Spencer, JAOCS 50:31 (1973).
18. Rackis, J.J., D.H. Honig, D.J. Sessa and H.A. Moser, Cereal Chem. 49:586 (1972).
19. Tallent, W.H., J. Harris, I.A. Wolff and R.E. Lundin, Tetrahedron Lett. 1966:4329.
20. Morris, L.J., R.T. Holman and K. Fontell, JAOCS 37:323 (1960).
21. Veldink, G.A., G.J. Garssen, J.F.G. Vliegenthart and J. Boldingh, Biochem. Biophys. Res. Commun. 47:22 (1972).
22. Leu, K., and C. Eriksson, Presented at the ISF XI World Congress, Göteborg, Sweden, June 1972.
23. Heimann, W., and P. Schreier, Helv. Chim. Acta 53:2296 (1970).

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Changes in Lipid Composition of Germinating Barley Embryo

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ABSTRACT

Barley seeds, *Hordeum vulgare*, var. Kenia, were dissected before and after 5 days of germination, to distinguish between the scutellum, the coleoptile half of the embryo and the coleorhiza half of the embryo. Total lipids were extracted from each fraction and analyzed by thin layer chromatography and gas liquid chromatography. In tissues from the coleoptile and coleorhiza halves of the embryo there was a concurrent disappearance of triglycerides with a marked increase of esterified sterols and esterified sterol glucosides. In the scutellum there was also a change in triglycerides, but the variations in contents of esterified sterols and esterified sterol glucosides were much smaller. Mono- and digalactolipids were virtually absent from embryonic tissue. The amounts of linoleic and linolenic acids in esterified sterol glucosides were increased after 5 days of germination in all the embryonic tissues, especially in the coleoptile half. In sterol esters, linoleic acid comprised nearly half of the total fatty acids, and the desaturation after 5 days of germination was much less pronounced.

INTRODUCTION

Lipids are known to have several functions in seeds: as a reserve energy source, e.g., oil seeds; as necessary components of membrane systems (1,2), and as emulsifiers of fat substrates in vivo for lipases (3). Barley contains 2% total lipids of which one-third is in the embryo, which represents less than 3% of the weight of the seed (4,5). This metabolically active tissue containing one-third of the total lipids of the seed suggests that embryo lipids might have several important functions for the proper germination of barley. However a complete distribution of the classes of lipids in embryonic tissues has not yet been reported.

The purpose of this investigation was to determine the lipid classes and the fatty acid distribution in barley embryo, as well as to identify the changes in these lipid components of the scutellum, and of the coleoptile (ED) and the coleorhiza (EF) halves of the embryo from ungerminated and 5 day germinated seeds.

MATERIALS AND METHODS

Seed

Two-row barley, *Hordeum vulgare*, var. Kenia, was obtained from the Carlsberg Brewery Lab., Copenhagen.

Reagents

All reagents were analytical grade. Solvents used for thin layer chromatography (TLC) were distilled before use. Standards for TLC identifications were obtained as follows: cholesterol palmitate, methyl-linoleate, triolein and linoleic acid from The Hormel Institute, Austin, Minn.; cholesterol (F. Hoffmann-La Roche & Co., Ltd., Basle, Switzerland) was further purified by recrystallization from alcohol. Phosphatidyl choline was prepared from crude egg lecithin (Sigma type IIE) by preparative TLC. Phosphatidyl ethanolamine (PE) from pig liver was isolated by column chromatography and purified by preparative TLC.

Mono- (MGDG) and digalactodiglycerides (DGDG) were prepared from spinach leaves. Lipid extraction was made according to Folch

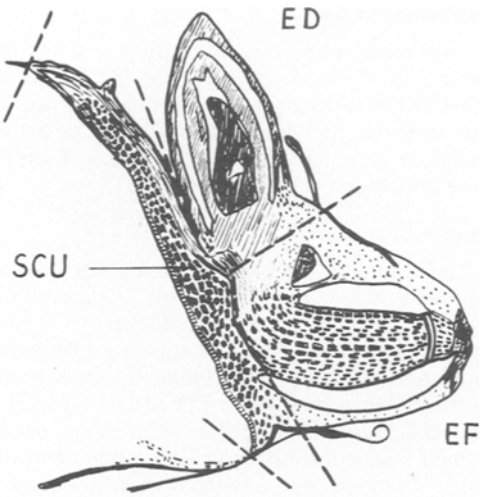

FIG. 1. Barley embryo of a soaked grain. Dissecting lines are given (- - - -).

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TABLE I

Weight of Dried Tissues and Recovered Lipids from Barley Embryo after 0 and 5 Day Germination[a]

Sample	Tissue, dry wt,mg			Total lipid extracted, mg			Total lipid, % dry tissue		
	A	B	C	A	B	C	A	B	C
Coleorhiza-0	49.5	44.9	46.2	8.5	8.6	10.4	17.2	19.2	22.6
Coleoptile-0	24.1	22.6	20.2	7.1	3.5	4.6	29.6	15.6	22.6
Scutellum-0	76.9	79.2	79.4	18.6	13.8	14.8	24.2	17.5	18.6
Coleorhiza-5	55.3	54.3	41.4	5.1	3.8	5.1	9.2	7.0	12.3
Coleoptile-5	34.0	27.9	28.1	2.2	2.7	2.6	6.4	9.5	9.3
Scutellum-5	86.2	82.1	93.4	13.4	14.2	15.1	15.6	17.2	16.0

[a]For 5 day germinated seeds dissected coleoptile length was 3-5 cm. Root length was 5-7 cm. Each weight is for 100 seeds.

et al. (6). Chromatography of 160 mg of the extracted material was performed on a column containing 60 g silicic acid (Baker Analyzed Reagent, Deventer, The Netherlands) and 20 g Hyflo Supercel (Johns Manville Co., New York) prewashed with 200 ml chloroform. Elution was made by a modification of the system described by Rosenberg et al. (7): (a) 360 ml chloroform removed carotenoids, pigments and nonpolar lipids, such as triglycerides; (b) 320 ml chloroform-acetone 1:1 v/v in 40 ml portions (fractions B1-B8) eluted the MGDG, and fractions B5-B7 contained pure MGDG, as indicated by TLC; (c) 320 ml acetone released the DGDG. Fractions C5-C8, containing the major part of the DGDG, were shown by TLC to be very pure.

Germination of Seeds

Dry seeds were presoaked in ice water for 5-6 hr, then placed between double sheets of moist filter paper in large covered Petri dishes. The seeds were kept moist during the 5 day period of germination at 19 C in dark, temperature-controlled walk-in incubators.

Dissection of Tissues

Ungerminated and germinated seeds, in 100 seed lots, were dissected under a microscope according to the diagram in Figure 1. In this way the scutellum could be separated from the embryo, which was then divided through the internode into the distal ED and the forward EF. To insure the removal of as much newly formed leaf and root tissue as possible from the 5 day embryos, the new growth was dissected free at the edge of the scutellum, as illustrated by the dotted lines in Figure 1.

Extraction of Lipids

After dissection, the six lots of tissues were dried in vacuo to constant weight over P_2O_5 in a desiccator in the refrigerator for 7-14 days. Each was then extracted in Potter-Elvehjem glass homogenizers three times in chloroform-methanol 3:1 at 55 C, twice in ether, then once in a 1:1 mixture of the two solvents. The combined extracts of total lipids from each tissue were washed in 0.73% NaCl, then water, dried with anhydrous Na_2SO_4, filtered, and the solvents removed under nitrogen and mild heat (30-35 C). The lipid residues were each made up to a final concentration of ca. 3 mg/100 μl chloroform-methanol 2:1.

Analysis of Lipids by Thin Layer Chromatography

TLC was performed on standard plates, 20 x 20 or 10 x 20 cm, with a migration of 15 cm in a saturated chamber. For separation of neutral lipids (NL), Silica Gel G (0.250 mm) and solvent systems containing petroleum ether-diethyl ether-acetic acid 80:20:1 v/v/v or 70:30:1 v/v/v were used.

Polar lipids (PL) were chromatographed on Silica Gel H (0.5 mm) in a system containing chloroform-methanol-water 50:20:3 v/v/v. Spots were located and partially identified by spraying with 50% H_2SO_4 and subsequent charring. Gentle heating with sulfuric acid gave red spots for sterol-containing substances and more violet spots for glycolipids.

Among the PL, identification of aminophosphatides was made by ninhydrin spray, and choline-containing lipids by a modified Dragendorff reagent as described by Wagner et al. (8).

Separation of MGDG and DGDG from phospholipids was obtained using a solvent system containing acetone-acetic acid-water 100:2:1 v/v/v (9).

In an attempt to identify some of the unknown spots in TLC, small scale preparative TLC on common 0.25 mm plates was employed. The lipid zones were scraped off the plates and transmethylated directly with dry

hydrochloric acid in dry methanol (10). The components released from the lipid sample after methylation were examined by TLC in a solvent system of petroleum ether-diethyl ether-acetic acid 80:20:1. Further fractionation of the PL was obtained with the solvent system diisobutyl ketone-acetic acid-water 8:5:1 v/v/v, as described by LePage (11).

Analysis of Fatty Acids by Gas Liquid Chromatography

Methyl esters prepared as described above were analyzed on a Beckman GC4 gas chromatograph on a 6 ft x 1/8 in. stainless steel column containing 15% DEGS on 80/100 mesh Chromosorb W (AW) (Applied Science Labs.); column temperature 175 C; inlet temp., 240 C; detector temp. 240 C; carrier gas: helium, 75 ml/min. Standard mixture no. H 104 (Applied Science Labs.) and other known fatty acid methyl ester mixtures containing unsaturated fatty acids were used for identification of the peaks. The logarithmic plot of retention times versus C-number was used for tentative identification where standards were not available.

Quantitation of Lipid Classes

The small amounts of lipid available necessitated a sensitive method for determination of the lipid classes. The large amount of triglycerides (TG) caused further complications. A method based on addition of an internal standard to the various scrapings from the TLC plates before transmethylation of the fatty acids was therefore used.

Pentadecanoic acid (The Hormel Institute) was chosen as internal standard, since this acid was present only as a minor compound in barley embryonic lipids.

For calculations of the amount of a certain lipid class, an average molecular weight for fatty acid methyl esters of 294.5 (corresponding to linoleic acid) was employed, and for the sterol-containing lipids the molecular weight of β-sitosterol, 414.7, was applied.

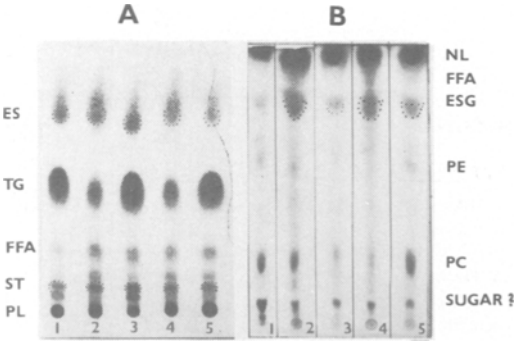

FIG. 2. Thin layer chromatographic separation of total lipids from embryo and scutellum of barley after 0 and 5 day germination. A, separation into esterified sterols, triglycerides, free fatty acids, sterols and polar lipids at origin; B, separation of polar lipids. 1: coleoptile-0; 2: coleoptile-5; 3: coleorhiza-0; 4: coleorhiza-5; 5: scutellum-5. Dotted lines indicate sterol-containing lipids. Solvent systems: A, petroleum ether-ether-acetic acid 80:20:1 v/v/v; B, chloroform-methanol-water 50:20:3 v/v/v.

RESULTS

Total Lipids

The results described are based on three separate 100 seed lots of 0 and 5 day germinated seeds. The figures in Table I represent recoveries of total lipids and weight of dry tissues. Values for total intact embryos reported by MacLeod and White (4) correspond to those obtained after summation of values for dissected tissues.

The dried weights of scutella from germinated seeds did not vary as much from the values of ungerminated seeds as might have been expected. Though the size of the scutellum increases three- or four-fold during germination, dry weights of tissues (100 seeds) only

TABLE II

Distribution of Lipid Classes in Embryonic Tissues of Barley[a]

Lipid class	Per cent					
	Coleorhiza-0	Coleoptile-0	Scutellum-0	Coleorhiza-5	Coleoptile-5	Scutellum-5
Esterified sterols	7.1	5.3	1.1	15.4	11.8	3.7
Free fatty acids	2.0	3.6	0.5	8.4	12.1	2.8
Triglycerides	65.2	58.1	86.3	55.1	39.7	71.4
Phosphatidyl choline	16.4	22.5	6.2	5.5	9.6	8.8
Phosphatidyl ethanolamine	5.2	4.7	2.5	3.2	9.6	4.7
Esterified sterol glucosides	4.1	5.8	3.4	12.3	17.1	8.6

[a]Percentages of fatty acid-containing lipids. 0 and 5 day germination.

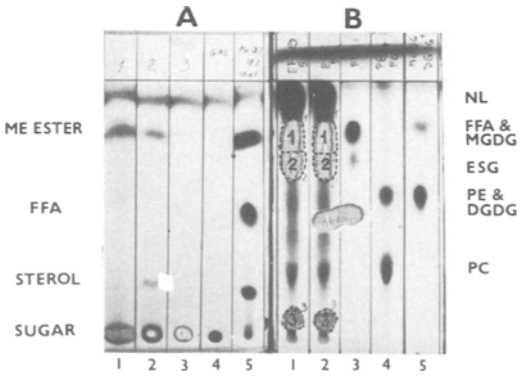

FIG. 3. Preparative thin layer chromatography of polar lipids from coleorhiza after 5 day germination (B) and identification after methanolysis (A). 3B: Runs 1 and 2: numbered fractions are scraped off and methylated. Fraction 1: free fatty acids + monogalactodiglycerides (if present); fraction 2: esterified sterol glucosides; fraction 3: sugar. Run 3: standard free fatty acids. Run 4: standard phosphatidyl ethanolamine + phosphatidyl choline. Run 5: standard monogalactodiglycerides + digalactodiglycerides. 3A: Run 1: fraction 1. Run 2: fraction 2. Run 3: fraction 3. Run 4: standard galactose. Run 5: standard methyl linoleate + linoleic acid + cholesterol. Solvent systems: A, petroleum ether-ether-acetic acid 80:20:1 v/v/v; B, chloroform-methanol-water 50:20:3 v/v/v.

increased ca. 10%, suggesting that most of the increase may be simply water expansion.

The total lipid contents of EF and ED as percentages of the dry tissue were drastically reduced during the 5 day germination, whereas the weights of the tissues were slightly increased. The total lipid content of the scutellum was much less influenced.

Lipid Classes in Barley Embryos

Figure 2A and B shows the distribution of lipid classes in the various embryonic tissues, and the percentages of total lipids (containing fatty acids) are given in Table II.

A marked decrease in TG from 0 to 5 days germination can be observed for both ED and EF (Table II and Fig. 2A), as well as for the scutellum. These data can be correlated with the findings of MacLeod and White (12) that two-thirds of the lipase activity of barley seeds is present in the embryo. The free fatty acid (FFA) content in our study increased slightly during the 5 day germination, but the amounts found do not account for the decrease in TG. As the total lipids decrease there is definite catabolism taking place. It appears that the FFA level in germinating seeds may reach a certain point and not exceed it. This has been noted in some oilseeds, in which rather low levels of FFA were found after germination (13,14).

The free sterol contents are rather high in all of the barley samples tested (Fig. 2A). However the most striking observation is the increase in esterified sterols (ES) within 5 days. There is an unidentified tailing spot above ES that gives no sterol color with H_2SO_4 and heat. It is present in both EF and ED samples after 5 days, but is nearly absent in the scutellum and may possibly be waxes extracted from the sheaths covering the coleoptile and coleorhiza. Except for the husks, there have been very few reports on wax contents in barley. No further identification of the waxes was made in this study.

Among the changes detected in PL (Table II and Fig. 2B) during germination, a marked increase in esterified sterol glucosides (ESG) was the most pronounced, especially for EF and ED tissues.

The amounts of PC in EF and ED tissues seemed to decrease when determined as percentage of total lipids, whereas the scutellum maintained or even increased the amount. On the contrary, the PE are nearly unchanged except in the coleoptile, where an increase could be observed. In general, the contents of polar lipids were higher in ED tissue than in EF tissue.

Changes in the lipid composition of whole barley seedlings during malting were reported by Holmberg and Sellman-Persson (15). The proportion of lipids was not altered much, except for the PL which sometimes decreased from ca. 21% to 4%. However some experimental malts were unaffected. From their results, although not pointed out, it appears that the contents of sterol and wax esters increase considerably in the total grain during malting. This is similar to the changes observed in ES in our study of barley embryo tissues during 5 day germination.

The solvent system used to develop the TLC plates in Figure 2B does not separate MGDG from ESG or PE from DGDG. Thus smaller amounts of such galactolipids might have been masked. In a solvent system of acetone-acetic acid-water, however, phospholipids do not migrate on the TLC plate, and the occurrence of MGDG and DGDG in the tissues could be ascertained. This system revealed the absence of MGDG and DGDG in all samples examined, except trace amounts in the coleoptile part of the embryo, although galactolipids have been reported present in cereal grain flours (16). The results are, however, in accordance with findings for wheat that galactolipids are located in the endosperm (17). The trace amounts of galactolipids in ED tissues are possibly derived from chloroplasts normally containing galactolipids (18).

Characterization of Esterified Sterol Glucosides

For further identification of the ESG, these spots were scraped from the TLC plates and methylated as described above. Three fractions were obtained (Fig. 3B).

Figure 3A shows that fraction 1 contained one spot migrating in a manner similar to that of the standard fatty acid methyl ester and one which did not migrate in the solvent system used. The discolored band near the front of the three fractions in Figure 3A was caused by the omission of a prewashing step for this plate. This band is absent in Figure 3B because the prewashing was carried out. Fraction 1 therefore appears to be a mixture of FFA and possibly MGDG, if present. The correlation with R_f values for standard substances in Figure 3B supports this interpretation.

Fraction 2 yielded three spots; the first migrates as the standard fatty acid methyl ester, the second as a sterol, and the third does not move in the solvent employed. This latter spot agress in R_f with the standard galactose. Fraction 2 is therefore considered to be ESG. Fraction 3 showed only one spot which remained at the origin and was identified as a carbohydrate.

Fatty Acid Distribution in Embryonic Tissues before and after Germination

The results from gas liquid chromatographic analyses are given in Table III.

Triglycerides

In ungerminated seeds there are only small differences in the fatty acid pattern of EF, ED and the scutellum, suggesting that the oil contained in spherosomes, the primary site of TG storage in seeds, is the same regardless of the tissue. The main component, ca. 45-50%, is linoleic acid, but palmitic, oleic and linolenic acids are also major components (10-25%). The amount of stearic acid, in contrast, is very small, ca. 1%. The scutellum seems to have a somewhat lower content of linolenic acid. During 5 day germination there is a very distinct decrease in the amount of TG. The variation in fatty acids, however, is much smaller, but there seems to be a desaturation of the C-18 acids, especially in EF and ED tissues. In addition, these two tissues seem to differ in their respective products of desaturation; the coleoptile thus having the greater amount of unsaturated fatty acids. This may be due to the presence of prochloroplasts in ED tissues.

Esterified Sterol Glucosides

This lipid class is much more saturated than the TG, especially for the ungerminated tissues. In dry embryonic tissues, palmitic and linoleic acids are the major components, both comprising ca. 20% of the fatty acids. Stearic and oleic acids are also present in significant quantities, whereas linolenic acid only ranges from 3 to 7% of the total fatty acids. After germination, there is a distinct change in this fatty acid pattern with a marked increase in the more unsaturated C-18 fatty acids, even greater than that observed for the triglycerides. As seen for TG, this process is more prevalent in ED tissue compared to EF. The variations in scutellum fatty acids generally follow the same patterns but are less pronounced.

LePage (11,19) has reported high amounts of ESG in potato tubers (10%) and shown that it contained equal amounts of sterol, glucose and fatty acids. Fatty acids were attached to the C-6 of the sugar and sterols at the C-1. The fatty acid composition of ESG in potato tubers showed 50-60% palmitic acid as the major component, with 20-25% linoleic acid as well as a small quantity of linolenic acid. The latter findings are in line with our results on ungerminated barley embryos, although in barley saturated acids were distributed between C-14:0, C-16:0 and C-18:0.

Sterol Esters

Sterols in embryonic tissue are predominantly esterified to unsaturated fatty acids, especially C-18:2ω6 which comprises about half of the total. The total amount of saturated acids is less than 20%. The fatty acid spectrum of ES resembles the triglycerides more than it does the ESG, which seems to indicate the lack of a direct relationship between ESG and ES. The molecular configuration of ESG, in which the fatty acid is esterified at the 6 position in glucose, also tends to discount such a relationship. However the compositions of the ES and the free sterols show a relationship. The function of ES in barley embryo might therefore be, as was shown for animal systems (20), the transport of sterols and fatty acids released from TG for further synthesis and for membrane formation. The role of ES as a sterol carrier was also discussed by Dupéron (21).

For all of the lipid classes identified, it was observed that various parts of the embryo have different fatty acid compositions which are probably related to their different functions. However it could also be a reflection of the abundance of various organelles with specific membrane structures containing varying lipid compositions.

The increase in ES and ESG of barley embryonic tissue after 5 day germination may thus affect two different aspects of seed devel-

TABLE III

Fatty Acid Composition of Triglycerides, Esterified Sterol Glucosides and Sterol Esters from Barley Embryonic Tissues[a]

Fatty acid	Triglycerides						Esterified sterol glucosides						Esterified sterols					
	Coleorhiza		Coleoptile		Scutellum		Coleorhiza		Coleoptile		Scutellum		Coleorhiza		Coleoptile		Scutellum	
	0	5	0	5	0	5	0	5	0	5	0	5	0	5	0	5	0[b]	5
14:0	0.4	0.5	0.3	0.3	0.2	0.3	5.4	1.9	7.4	1.4	10.5	6.1	1.3	4.2	5.5	2.4	14	3.1
16:0	21.4	16.5	19.2	15.7	23.5	22.5	24.9	23.9	31.8	26.2	30.2	35.2	6.7	11.8	14.3	9.3	15	8.4
16:1ω7	0.8	1.0	0.8	1.6	0.5	1.0	5.6	2.4	5.5	0.9	5.9	2.9	4.1	7.9	6.9	8.2	7	4.3
16:2	—	—	—	0.3	—	—	1.4	2.0	2.7	1.1	1.4	Trace	0.6	1.8	Trace	Trace	Trace	1.3
(17:0)																		
17:1	—	—	—	—	—	—	3.1	—	2.9	0.8	3.9	0.9	1.0	1.9	3.4	2.1	5	1.4
18:0	0.9	1.2	0.8	1.1	0.6	1.0	13.2	6.2	14.0	1.7	9.5	4.6	1.9	2.0	3.2	3.5	6	6.5
18:1ω9	15.0	10.4	17.7	11.9	14.4	11.7	13.3	7.4	14.0	5.7	13.1	5.2	8.4	8.4	14.5	16.1	12	8.0
18:2ω6	46.9	54.3	47.0	47.8	52.0	51.8	20.1	40.2	15.2	39.9	21.7	29.9	59.2	49.7	38.5	42.5	36	50.2
18:3ω3	12.4	12.2	11.4	17.7	7.3	9.5	7.2	14.6	3.4	20.7	3.7	13.2	11.8	7.9	6.8	11.6	4	6.7
20:0	—	—	—	—	—	—	3.3	—	—	—	—	—	—	—	—	—	—	—
20:1	1.9	2.3	2.2	2.3	1.2	2.1	—	Trace	3.1	1.7	—	Trace	2.9	1.0	3.0	4.2	2	4.6
20:2	—	Trace	—	—	—	—	2.5	0.7	Trace	Trace	—	—	1.0	Trace	—	—	—	—
22:0	—	—	—	—	—	—	—	0.9	—	—	—	—	0.6	Trace	1.2	—	—	—
22:1	0.4	0.7	0.6	1.3	0.2	Trace	—	—	—	—	—	—	—	—	—	—	—	—

[a]Wt% of total fatty acids. 0 and 5 day germination.

[b]Only very small amounts available.

opment: (a) Sterol esters act as carriers of sterol and fatty acids to the growing areas of the seedling, and (b) esterified sterol glucosides, with both polar and nonpolar regions of the molecule, seem ideally suited as membrane constituents; but a role as transport intermediates cannot be excluded.

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REFERENCES

1. Van Deenen, L.L.M., JAOCS 43:296 (1966).
2. Fleischer, S.F., and H. Klouwen, Biochem. Biophys. Res. Commun. 5:378 (1961).
3. Ory, R.L., A.J. St.Angelo, I.V. DeGruy and A.M. Altschul, Can. J. Biochem. 45:1445 (1967).
4. MacLeod, A.M., and H.B. White, J. Inst. Brew. 67:183 (1961).
5. Banasik, O.J., and K.A. Gilles, Cereal Sci. Today 11:98 (1966).
6. Folch, J., M. Lees and G.H. Sloane-Stanley, J. Biol. Chem. 226:497 (1957).
7. Rosenberg, A., J. Gouaux and P. Milch, J. Lipid Res. 7:733 (1966).
8. Wagner, H., L. Hörhammer and P. Wolff, Biochem. Z. 334:175 (1961).
9. Gardner, H.W., J. Lipid Res. 9:139 (1968).
10. Stoffel, W., F. Chu and E.H. Ahrens, Anal. Chem. 31:307 (1959).
11. LePage, M., Lipids 3:477 (1968).
12. MacLeod, A.M., and H.B. White, J. Inst. Brew. 68:487 (1962).
13. Ching, T.M., Plant Physiol. 38:722 (1963).
14. St.Angelo, A.J., and A.M. Altschul, Ibid. 39:880 (1964).
15. Holmberg, J., and G. Sellmann-Persson, Eur. Brew. Convent. Proc. 1967:213.
16. Carter, H.E., K. Ohno, S. Nojima, C.L. Tipton and N.Z. Stanecev, J. Lipid Res. 2:215 (1961).
17. De Stefanis, V.A., and J.C. Ponte, Jr., Biochim. Biophys. Acta 176:198 (1969).
18. Bamberger, E.S., and R.B. Park, Plant Physiol. 41:1591 (1966).
19. LePage, M., J. Lipid Res. 5:587 (1964).
20. Heftmann, E., Lipids 6:127 (1971).
21. Dupéron, P., Physiol. Vég. 9:373 (1971).

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Regulation of Hepatic Cholesterol Synthesis from Mevalonate in Suckling and Weaned Rats

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ABSTRACT

The conversion of mevalonic acid into total nonsaponifiable lipids (NSF) and digitonin precipitable sterols (DPS) by 5000 g liver supernatant fractions was compared in suckling and weaned rats. The incorporation of mevalonate into both NSF and DPS was low in fractions from suckling rats and very high in fractions from weaned rats. The results indicate that the activities of one or more of the enzymes catalyzing the conversion of mevalonate into squalene and squalene into cholesterol change after weaning and may act as regulatory step(s) for cholesterol synthesis in the livers of suckling rats. It is suggested that the reduced synthesis of cholesterol in the livers of suckling rats is caused by the cholesterol in the maternal milk, and the rapid rise in cholesterol synthesis after weaning is due in part to the dietary change accompanying weaning, and in part to an increased need for cholesterol by developing liver.

INTRODUCTION

Hepatic synthesis of cholesterol in the matured animal is sensitive to a variety of nutritional and experimental conditions. Although synthesis of cholesterol in adult liver is regulated primarily at the HMG CoA reductase step, Gould and Swyryd (1) and Bucher et al. (2) have reported data indicating that there are secondary regulatory sites in the synthesis of cholesterol from mevalonic acid. There is evidence that hepatic cholesterol synthesis changes during development (3,4). The change in rat hepatic HMG CoA reductase activity during development (5) suggests that this enzyme may regulate hepatic cholesterol synthesis during development as well as in the adult animal. In the present study we have compared the conversion of mevalonic acid into cholesterol by cell free preparations from livers of suckling and weaned rats, in order to determine whether or not hepatic cholesterol synthesis in suckling rats is regulated beyond the mevalonate step. The incorporation of mevalonate into nonsaponifiable lipids and digitonin precipitable sterols was found to be moderately high in the 1st week of postnatal life, very low during the 2nd and 3rd weeks and very high after weaning. These results suggest that the activities of enzymes in the multistep conversion of mevalonate to cholesterol in the liver also changed during development, and may have a regulatory role in hepatic cholesterol synthesis in the immature animal.

MATERIALS AND METHODS

DL-2[^{14}C] mevalonic acid as the dibenzylethylene diamine (DBED) salt was purchased from New England Nuclear Corp., Boston, Mass. Unlabeled mevalonic acid DBED salt was obtained from K and K Laboratories, Inc., Plainview, N.Y. Pups were separated from the mother at 20 days of age, suckling rats were less than 20 days old and weaned animals were 22-40 days old. The diet of the mother and of weaned pups consisted of laboratory chow from Ralston Purina Co., St. Louis, Mo.

Livers obtained from two to six Sprague Dawley rats (sacrificed between 8 and 10 A.M.) were homogenzied with thee volumes of 0.02 M potassium phosphate buffer containing 0.1 mM EDTA. Homogenates were centrifuged at 5000 g for 15 min in an International Centrifuge Model T60. The 5000 g supernatant fraction was incubated (a) with varying amounts of cell fraction and a fixed concentration of mevalonate, (b) with varying concentration of mevalonate and fixed amount of cell fraction, and (c) with a fixed amount of cell fraction and mevalonate but for varying time intervals. Incubations were carried out in an atmosphere of 95% O_2 and 5% CO_2 at 37 C. Each incubation contained, in addition to mevalonate and the cell fraction, 3 μmol ATP, 10 μmol magnesium chloride, 2 μmol NAD, 2 μmol NADP, 20 μmol glucose-6-phosphate, 2 units glucose-6-phosphate dehydrogenase and 60 μmol nicotinamide, in a final volume of 2.5 ml 0.02 M potassium phosphate buffer solution (pH 7.4). Incubations were terminated by addition of 15% KOH in 50% ethanol. Saponification, extraction of nonsaponifiable lipids (NSF) and precipitation of 3βhydroxy sterols by digitonin (DPS) were carried out as described elsewhere (6).

When the nonsaponifiable lipid fraction was separated by thin layer chromatography (TLC) on Silica Gel G (6), two major radioactive peaks occurred, which corresponded to squalene and

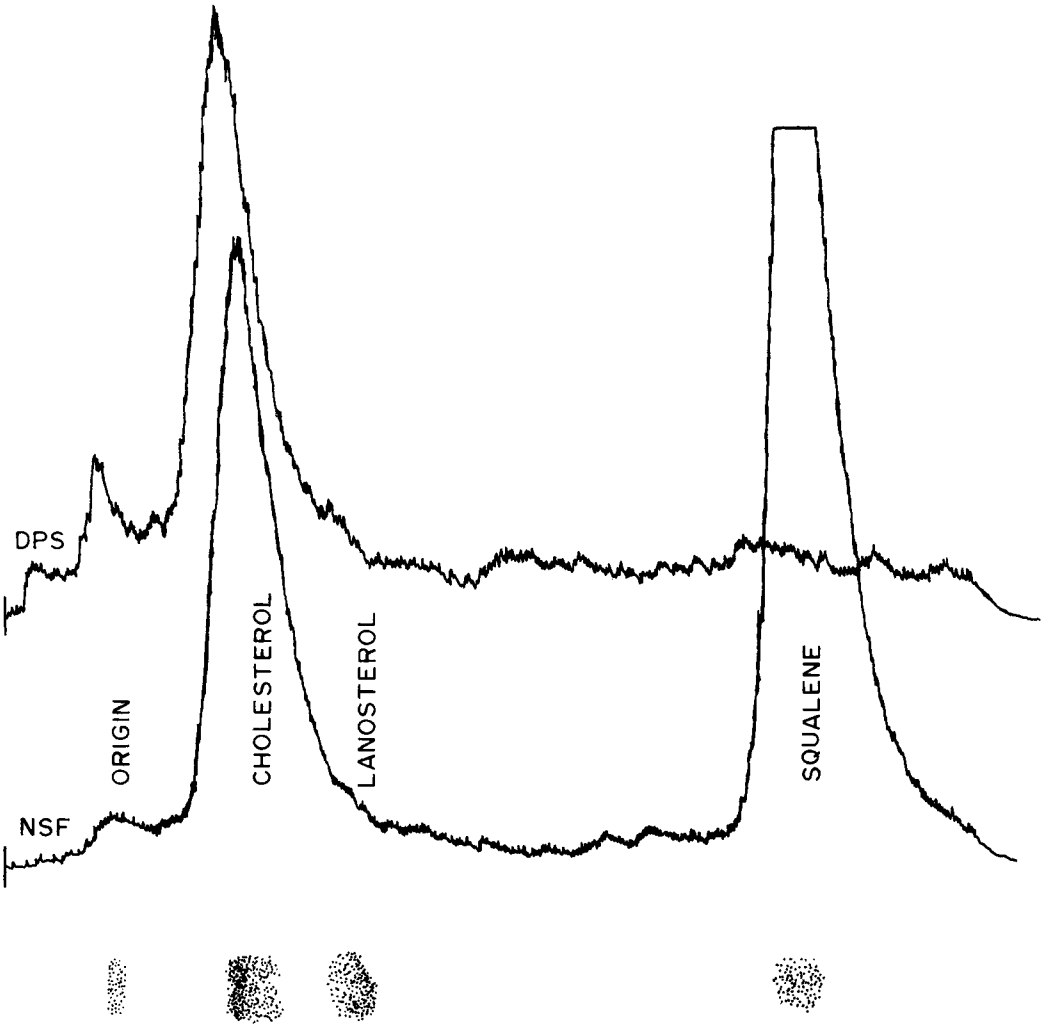

FIG. 1. Radioactivity scan of nonsaponifiable lipids (NSF) and digitonin precipitable sterols (DPS). Portions (0.2 ml) of 5000 g supernatant from livers of 23- to 25-day-old rats were incubated with mevalonic acid-2[^{14}C] DBED salt (3.0 x 10^5 cpm) and 250 nmoles unlabeled mevalonate for a period of 2 hr. NSF and DPS fractions were prepared from incubates as described in text and subjected to thin layer chromatography on Silica Gel G.

cholesterol (Fig. 1). Separation of the digitonin precipitable sterols gave a single radioactivity peak that corresponded to cholesterol. Digitonin precipitates were dissolved in 0.5 ml pyridine, to which 5 ml water was added. Sterols were then extracted by ethyl ether and separated by TLC.

RESULTS

The incorporation of mevalonate into total nonsaponifiable lipids (NSF) and digitonin precipitable sterols (DPS) by a 5000 g supernatant fraction from livers of suckling (7- and 15-day-old) and weaned (24- and 36-day-old) rats is shown in Figure 2. The amount of mevalonate converted to NSF increased with increasing amounts of the supernatant fraction (Fig. 2). The incorporation was moderately high in the liver preparations from 7-day-old rats. There was a significantly lower conversion of mevalonate to NSF in fractions derived from 15-day-old rats. In fractions from weaned rats (24- and 36-day-old) the incorporation of mevalonate into NSF was much higher than that observed with either of the two suckling periods (7- and 15-day-old). The recovery of mevalonate into DPS was also slightly higher in fractions from 7-day-old rats than in those from 15-day-old rats, and highest of all in rats that

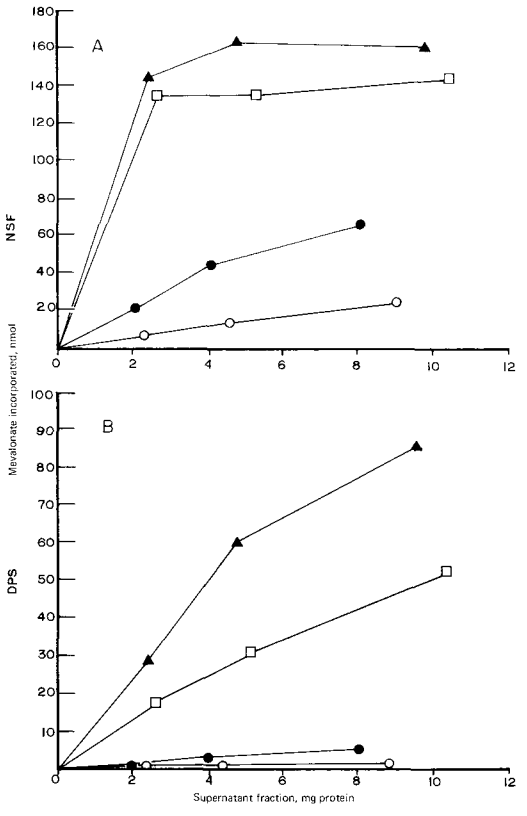

FIG. 2. Incorporation of mevalonate into total nonsaponifiable lipids (NSF) and digitonin precipitable sterols (DPS), by 5000 g supernatant fraction from livers of suckling and weaned rats. Varying amounts of supernatant fractions obtained as described in the text were incubated with mevalonic acid-2-[^{14}C] DBED salt (3.0 x 10^5 cpm) 250 nmol unlabeled mevalonate for a period of 2 hr. Other details of incubation conditions are described in text. Each point in figure is average of closely agreeing duplicate determinations. A, NSF; B, DPS, ● - ●, 7-day-old; ○ - ○, 15-day-old; ▲ - ▲, 24-day-old; □ - □ 36-day-old.

had just been weaned (24-day-old, Fig. 1). The data in Figure 3 show incorporation of mevalonate into NSF and DPS as a function of mevalonate concentration. Again the liver fractions from weaned rats (22- and 31-day-old) incorporated significantly higher amounts of mevalonate into both NSF and DPS than did the fraction from suckling rats (5- and 15-day-old). The data presented in Figure 4 indicated that rate of mevalonate incorporation into NSF and of NSF material to DPS is highest immediately after weaning (24- and 31-day-old) and lowest during the suckling period (6- and 13-day-old).

Data given in Figures 2-4 were rearranged in order to show the change during development

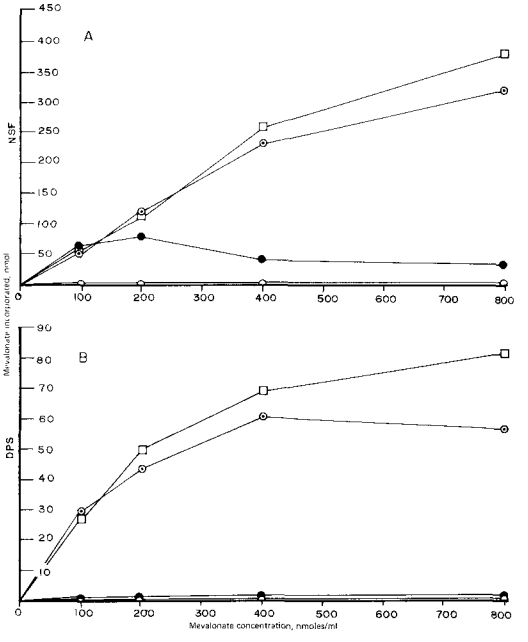

FIG. 3. Effect of mevalonate concentration on its incorporation into NSF and DPS. 0.2 ml portions of 5000 g supernatant fractions (5-6 mg protein) obtained from livers of respective age groups of rats were incubated with mevalonic acid-2-[^{14}C] DBED salt (3.0 x 10^5 cpm) and varying amounts of unlabeled mevalonate for a period of 2 hr. Other details of incubation conditions are described in text. Each point is average of closely agreeing duplicate determinations. A, NSF; B, DPS, ● - ●, 5-day-old; ○ - ○, 15-day-old; □ - □, 22-day-old; 0 - 0, 31-day-old.

of mevalonate incorporation into NSF and DPS as a function of age (Fig. 5). The change in liver weight with age is shown in Figure 6. Comparison of data in Figure 5 with that in Figure 6 indicates a close correlation between increase in cholesterol synthesis from mevalonate and increase in liver weight during development.

DISCUSSION

The results of separation of NSF by TLC indicated that radioactivity recovered from the NSF fraction is primarily associated with squalene and cholesterol. Separation of the digitonin-precipitable sterol fraction indicated that 80-90% of the radioactivity in this fraction was associated with cholesterol. Thus changes in the ratio of radioactivity in DPS to radioactivity in NSF reflect a change in the conversion of squalene to cholesterol. The DPS/NSF ratio calculated from Figure 1 was 0.03:0.08 for 7-day-old rats, 0.02:0.03 for 15-day-old rats, 0.2:0.5 for 24-day-old rats, and 0.1:0.45 for 34-day-old rats. These differences in the

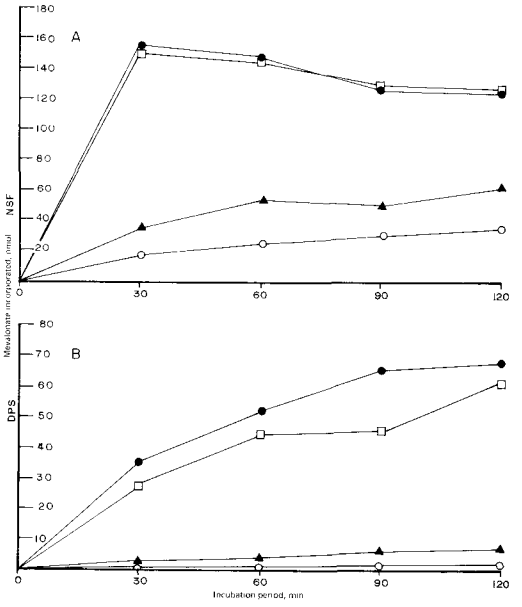

FIG. 4. Effect of incubation period on incorporation of mevalonate into NSF and DPS. 0.2 ml of 5000 g supernatant fraction (5-6 mg protein) from livers of rats of respective age groups were incubated with mevalonic acid-2-[^{14}C] (3.0 x 10^5 cpm) and 250 nmol unlabeled mevalonate for varying periods. Details of incubation conditions and cofactors are described in text. Each point is average of closely agreeing duplicate determinations. A, NSF; B, DPS; ▲ - ▲, 6-day-old; ○ - ○, 13-day-old; ● - ●, 24-day-old; □ - □, 31-day-old.

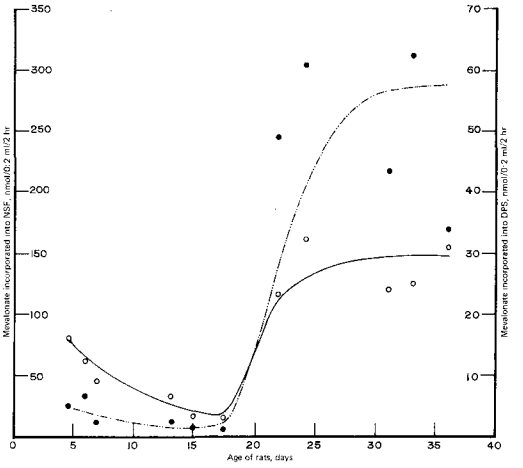

FIG. 5. Effect of age of rat on incorporation of mevalonate into NSF and DPS by 5000 g liver supernatant fraction. Data given in Figures 1-3 were rearranged to show change during development. ○ - ○, NSF; ● .-..-. ●, DPS.

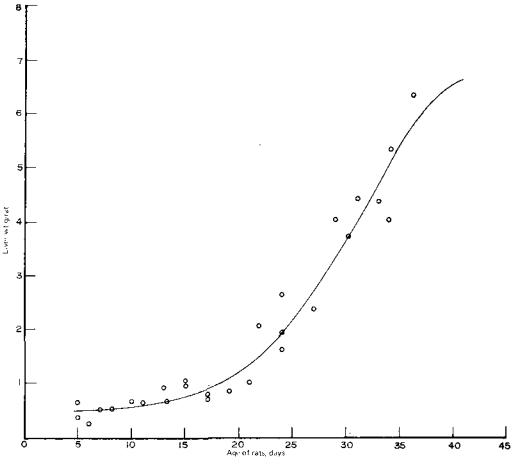

FIG. 6. Effect of age of rat on liver weight. Each point represents average weight of livers from 2-6 animals.

DPS/NSF ratio, along with the changes in the incorporation of mevalonate into NSF and DPS during development, suggest that both incorporation of mevalonate into squalene and incorporation of squalene into cholesterol change in the livers of suckling and weaned rats.

The developmental change in the capacity of cell free preparations from liver to incorporate mevalonate into NSF (squalene) and the latter to DPS (cholesterol) coincides with the reported developmental changes in HMG CoA reductase activity (5) and in hepatic cholesterol synthesis from glucose and acetate (3,4). Although reduction of HMG CoA to mevalonate may be a primary regulatory site of hepatic cholesterol synthesis in suckling and weaned rats, the fact that we found changes in both the incorporation of mevalonate into squalene and its further incorporation into digitonin precipitable sterols indicates that regulation of cholesterol synthesis in these animals also occurs beyond the mevalonic acid step.

Although it is generally accepted that hepatic cholesterol synthesis in adult animals is regulated at the HMG CoA reductase step, early studies of Bucher et al. (2) have shown that the conversions of both mevalonic acid and squalene to cholesterol by adult liver preparations are reduced by fasting or by feeding cholesterol. This suggests either that secondary regulatory mechanisms are important in controlling the flux of acetate through the entire pathway, or that whole segments of the pathway of synthesis may be depressed together. Later Carroll (3) demonstrated two secondary regulatory sites in cholesterol synthesis—one between mevalonate and farnesyl pyrophosphate and the other in the conversion of farnesyl pyrophosphate to squalene. Slakey et al. (7), from their

study of the effect of fasting and feeding on the levels of enzymes affecting the conversion of β-hydroxy-β-methylglutaryl CoA to squalene, have concluded that there are at least two secondary regulatory processes active in controlling conversion of mevalonate to cholesterol in the adult animal. The present data provides evidence that, as in adult rats, the synthesis of hepatic sterol in suckling rats may also be regulated between mevalonate and cholesterol.

McNamara et al. (5) reported low HMG CoA reductase activity in suckling rats and a rapid rise in activity following weaning. One reason why these authors feel that the low HMG CoA reductase activity in livers of suckling rats is not associated with cholesterol in milk is because the total hepatic cholesterol levels in the suckling rats are not different from adult levels. It should be pointed out, however, that the amount of cholesterol in the milk may be sufficient to provide the cholesterol needed for normal growth of liver tissue during the suckling period. Furthermore the developing liver may be more capable of adapting cholesterol synthesis to a changing requirement for cholesterol. The recent report of Reiser and Sidelman (8) supports this hypothesis. The rise in HMG CoA reductase activity following early weaning, the failure to observe the overshoot phenomenon characteristic of weaning at 21 days, and the fact that delayed weaning blocked the increase in the HMG CoA reductase activity (4) all suggest that the dietary change associated with weaning provides the primary stimulus for the reported increase in the HMG CoA reductase activity.

The postweaning increase in conversion of mevalonate to cholesterol could also be associated with the need for cholesterol by liver tissue, since growing liver tissue must synthesize its own cholesterol following weaning to diet low in fat and relatively free of cholesterol. It is of interest that a rapid decline in conversion of mevalonate to nonsaponifiables and digitonin precipitable compounds during fasting, and its return to normal after feeding a fat-free diet, parallels change in liver weight during fasting and refeeding (7). The question arises as to whether feeding a fat-free diet containing 1-2% cholesterol would eliminate the rise in cholesterol-synthesizing enzymes, which occurs during the feeding of a fat-free diet. Studies are in progress to determine whether weaning rats to a diet containing cholesterol and free of fat would prevent the rise in the capacity of liver tissue to convert mevalonate to cholesterol.

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REFERENCES

1. Gould, R.G., and E.A. Swyryd, J. Lipid Res. 7:698 (1966).
2. Bucher, N.L.R., K. McGarrahan, E. Gould and A.V. Loud, J. Biol. Chem. 234:262 (1959).
3. Carroll, K.K., Can. J. Biochem. 42:79 (1964).
4. Ballard, F.J., and R.W. Hanson, Biochem. J. 102:952 (1967).
5. McNamara, D.J., F.W. Quackenbush and V.W. Rodwell, J. Biol. Chem. 247:5805 (1972).
6. Shah, S.N., FEBS Letters 20:75 (1972).
7. Slakey, L.L., M.C. Craig, E. Beytia, A. Briedis, D.H. Feldbruegge, R.E. Dugan, A.F. Qureshi, C. Subbarayan and J.W. Porter, J. Biol. Chem. 247:3014 (1972).
8. Reiser, R., and Z. Sidelman, J. Nutr. 102:1009 (1972).

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I. Synthesis of *sn*-Glycerol-Cyclic-Phosphodiester Isomers[1,2]

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ABSTRACT

A procedure for the synthesis of stereochemically pure *sn*-glycerol-cyclic-phosphodiesters has been developed. The process involves the following sequence of reactions: benzyl-*sn*-glycerol→benzyl-*sn*-glycerol-cyclic(phenyl)-phosphodiester→ *sn*-glycerol-cyclic-phosphodiester. The following isomers have been synthesized: *sn*-glycerol-2,3-,1,2-, 1,3-cyclic-phosphodiesters and the racemic mixture. The 2,3- and 1,2-cyclic-phosphodiesters of glycerol are optically active antipodes. They are five-membered ring asymmetrical compounds, with specific rotations of $-1.6° \pm 0.1°$ and $+1.6° \pm 0.1°$, respectively. These two enantiomers and their racemate are thick liquids and are unstable; therefore they were converted into Ba(glycerol-cyclic-phosphodiester)$_2$ salts, which can be better stored. The six-membered ring *sn*-glycerol-1,3-cyclic-phosphodiester is a crystalline, stable compound. The physical and chemical properties of these cyclic-phosphodiesters of glycerol are described and their chemical analyses are reported.

INTRODUCTION

Half a century ago, Octave Bailly (1) reacted sodium phosphate with epichlorohydrin and claimed during reaction the formation of a six-membered ring cyclic-phosphodiester of glycerol takes place. About 20 years later, Verkade and coworkers (2) studied the acid-catalyzed phosphate group migration of glycerol-phosphoric-acid-ester and postulated a mechanism by way of cyclic-phosphodiester. Later, Chargaff (3) demonstrated that this rearrangement indeed involves an intramolecular migration of the phosphate group. Baer and Kates (4) studied phosphate group migration of synthetic *sn*-glycerol-3-phosphoryl-choline, in acidic and alkaline media, and also postulated the formation of the cyclic-phosphodiester of glycerol as an intermediate during the migration.

A similar phosphate group migration occurs during the hydrolysis of nucleic acid. Markham and Smith (5) have isolated and identified cyclic-2′,3′-nucleotides as intermediates in the hydrolysis of ribonucleic acid, and have shown that the formation of a cyclic-phosphodiester is responsible for the migration of the phosphate group from the 3′- to the 2′-hydroxyl of the ribose. Other cyclic-phosphodiesters have also been described, i.e., pantetheine-2′,4′-cyclic-phosphodiester (6), glucose-cyclic-phosphodiester (7) and riboflavine-4′,5′-cyclic-phosphodiester (8).

The possibility of isolation of a cyclic-phosphodiester of glycerol was investigated by Ukita et al. (9). They found that no evidence for accumulation of the cyclic-phosphodiester of glycerol during the hydrolysis of lecithin, but synthesized the cyclic-phosphodiester of glycerol by intramolecular cyclization of the *sn*-glycerol -2-phosphoric-acid-ester catalyzed with trifluoroacetic anhydride, according to the procedure of Brown and coworkers (10). Later, Maruo and Benson (11) prepared a radioactive glycerol-phosphate-ester by the method of McMurray et al. (12) and then cyclized this product by intramolecular phosphorylation (term introduced by Khorana et al. [13]) using dicyclohexylcarbodiimide (DCC) as catalyst, according to the procedure of Khorana and coworkers (13).

However no direct synthesis of isomerically pure cyclic-phosphodiesters of glycerol has been reported.

This paper describes the synthesis of the stereo- and positional isomers of *sn*-glycerol-cyclic-phosphodiester.

EXPERIMENTAL PROCEDURES

Benzyl Glycerol Ethers

Reaction of 2,3-isopropylidene-*sn*-glycerol and 1,2-isopropylidene-*sn*-glycerol with 50% sodium hydroxide yielded the sodium alkoxides as described by Kaufmann and Förster (14). Without isolation these were reacted with benzyl chloride and hydrolyzed according to the method of Sowden and Fischer (15) to produce 1- and 3-benzyl-*sn*-glycerols. 2-Benzyl-*sn*-glycerol was prepared from 1,3-benzylidene-*sn*-glycerol. The latter was prepared by the method of Hibbert and Carter (16), but with the modification introduced by Verkade and van Roon (17). 2-Potassium-1,3-benzylidene-*sn*-glyceroxide was prepared according to the method described by Gupta and Kummerow (18), and this, without isolation, was reacted with benzyl chloride to

[1] Presented in part at the ISF XIth World Congress, Göteborg, Sweden, June 1972.

[2] The nomenclature used in this communication is that adopted by IUPAC-IUB Commission.

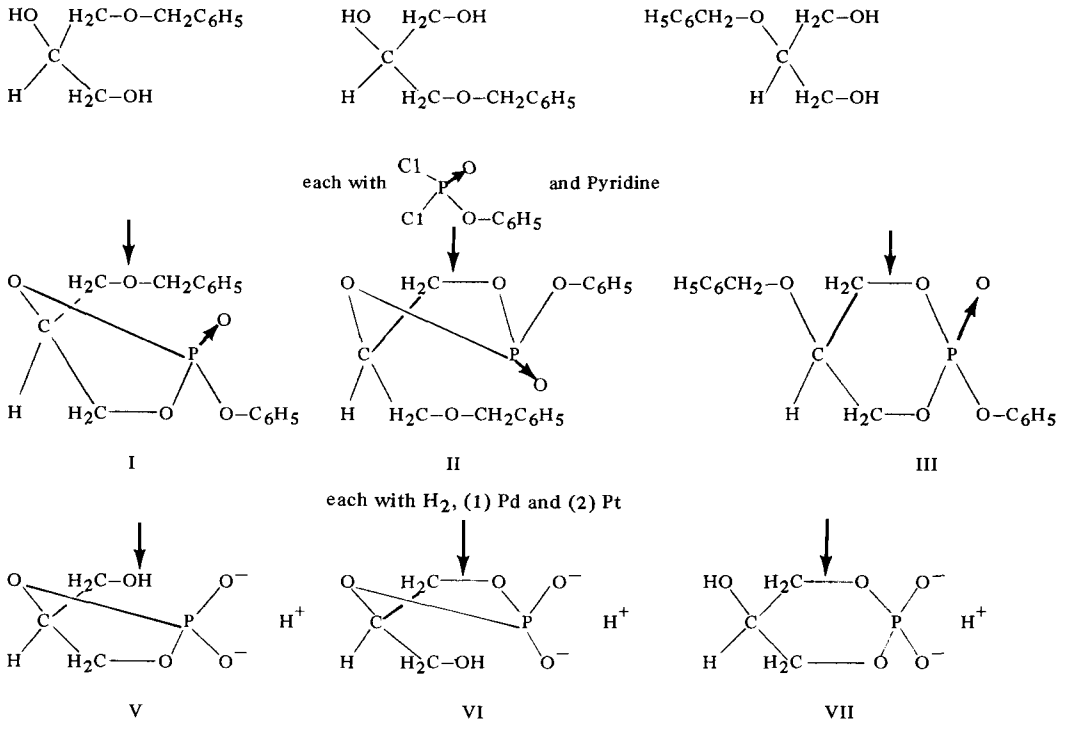


FIG. 1. Synthesis of *sn*-glycerol-cyclic-phosphodiester isomers.

produce 2-benzyl-1,3-benzylidene-*sn*-glycerol, which on hydrolysis of the benzylidene group with 10% acetic acid yielded 2-benzyl-*sn*-glycerol.

Benzyl chloride and phenylphosphoryl dichloride were certified reagents, and both were redistilled before use. Pyridine was dried over calcium hydride and toluene with sodium wire. Both solvents were certified spectroanalyzed.

Phosphocyclization

The phenylphosphoryl dichloride was used as phosphocyclizing reagent, as shown in the reaction scheme (Fig. 1). The position of the benzyl-protective group in the glycerol moiety dictates the cyclization position of the phosphodiester residue.

The phosphocyclization was carried out in a 500 ml three-necked, round-bottom flask fitted with a magnetic stirrer, two dropping funnels and a calcium chloride tube. The reaction flask was kept in a cold bath of ice and salt (-10 to -15 C). An appropriate benzyl-*sn*-glycerol (18.2 g, 0.1 mol) was dissolved in 100 ml dry pyridine (used as solvent and also as base to neutralize hydrogen chloride, which develops during phosphocyclization) and placed in one of the dropping funnels. An equimolar amount of phenylphosphoryl dichloride (21.0 g, 0.1 mol) was dissolved in dry toluene and brought to the same volume as the benzyl-*sn*-glycerol and pyridine mixture. The mixture of phenylphosphoryl dichloride and toluene was placed in the second dropping funnel.

Both reagents were added drop by drop at the same rate with stirring at -10 to -15 C in the reaction flask. The addition of the reagents was finished after ca. 30 min. The reaction mixture was stirred further for 2 hr at -10 C, and for 12 hr at room temperatue (20-25C). The reaction product was freed of the solvents, toluene and pyridine, by distillation in high vacuum at a bath temperature of 30-35 C. The residue, consisting of benzyl-*sn*-glycerol-cyclic(phenyl)-phosphodiester and pyridine hydrochloride, was dissolved in methanol or ethanol, and the alcoholic solution was then passed through an ion exchange column of Rexyn-101 (H^+) in order to remove the pyridine hydrochloride from the reaction product. The column was 40 cm long, 4.5 cm wide and contained 400 g Rexyn-101 (H^+).

The column was washed with either methanol or ethanol until the effluent was free of solute. The eluate was then concentrated to dryness under reduced pressure at a bath temperature of 30-35 C. The benzyl-*sn*-glycerol-cyclic(phenyl)-phosphodiester was recovered,

and, without further purification, showed on thin layer chromatography a major spot at R_f 0.7 and a very tiny spot at the origin, with benzene-diethyl ether 8:2 v/v used as developing solvent.

Two of the intermediate isomers (Fig. 1, structures I and II) and the racemic mixture, designated IV, were obtained in relatively high yields, i.e., over 90% of the theoretical yield. However another isomer (Fig. 1, structure III) was found to consist of two different compounds, which existed in a ratio of ca. 1:1, with total yield, as a mixture, of 88% of theory. The two compounds differed in their melting points, R_f values by thin layer chromatography and solubilities.

It seems reasonable to assume that the 2-benzyl-*sn*-glycerol-1,3-cyclic(phenyl)-phosphodiester appears in two different forms. The compound with high melting point (144-145 C) is suggested to be *trans*-form (IIIa), and the compound with lower melting point (71-72C) is suggested to be the *cis*-form (IIIb). Both 2-benzyl-*sn*-glycerol-1,3-cyclic(phenyl)-phosphodiester forms were crystalline, and were easily separated. Form IIIa crystallized from diethyl ether at 0 C, and form IIIb crystallized from petroleum ether (bp 30-60 C) at -6 C. These two forms could also be separated by column chromatography on silicic acid with benzene-diethyl ether 8:2 v/v as solvent, compound IIIa preceding compound IIIb in the elution.

Tentatively the structures, which are still under investigation are shown in Figure 2.

The analytical values of the stereoisomers and of the racemic mixture of benzyl-glycerol-cyclic(phenyl)-phosphodiester are listed below:

1-Benzyl-sn-glycerol-2,3-cyclic(phenyl)-phosphodiester (I): Specific rotation: $[\alpha]_D^{25}C + 7.5° \pm 0.1°$, in chloroform, c,10. Analysis calculated for $C_{16}H_{17}O_5P$(320): C, 60.00; H, 5.45; P, 9.67. Found: C, 60.12; H, 5.52; P, 9.60.

3-Benzyl-sn-glycerol-1,2-cyclic(phenyl)-phosphodiester (II): Specific rotation: $[\alpha]_D^{25}C$ $-7.5° \pm 0.1°$, in chloroform, c,10. Found: C, 59.89; H, 5.52; P, 9.84.

2-Benzyl-sn-glycerol-1,3-cyclic(phenyl)-phosphodiester (III): (a) *trans*-Form: mp 144-145 C. Found: C, 60.17; H, 5.84; P, 9.78. (b) *cis*-Form: mp 71-72 C. Found: C, 60.18; H, 5.35; P, 9.80.

Racemic benzyl-glycerol-cyclic(phenyl)-phosphodiester (IV): Found: C, 60.27; H, 5.44; P, 9.81.

The above isomers of benzyl-glycerol-cyclic(phenyl)-phosphodiester are readily soluble at room temperature in chloroform, ethanol, methanol, benzene and toluene, moderately soluble in diethyl ether and petroleum ether, and insoluble in water. At 20 C, I, II and IV are thick viscous liquids.

Catalytic Hydrogenolysis

Removal of benzyl-protective group with palladium black catalyst: Eleven grams of benzyl-glycerol-cyclic(phenyl)-phosphodiester was dissolved in 160 ml absolute ethyl alcohol by warming to 50 C. The clear solution, to which 2.5 g palladium black was added, was shaken in an all-glass reduction vessel in an atmosphere of pure hydrogen at room temperature and at a water pressure of 50 cm until the absorption of hydrogen ceased. The catalytic hydrogenolysis of the benzyl-protective group was completed at the end of ca. 1 hr, with enough uptake of hydrogen for ca. 100% removal of the benzyl-protective group. After replacing hydrogen by nitrogen, the reduction product was separated from the catalyst (palladium black) by centrifugation, and the catalyst was extracted three times with 25 ml portions of ethyl alcohol.

Removal of phenyl-protective group with platinum catalyst: The mother liquor, combined with ethyl alcohol extracts, together with 1.7 g platinum oxide, was placed in an all-glass reduction vessel, and the reductive cleavage was carried out as described above. The catalytic hydrogenolysis of the phenyl-protective group was completed at the end of ca. 3 hr, with consumption of ca. 4000 ml hydrogen (uncorrected). The hydrogen was then replaced with nitrogen, and the reduction product was separated from the catalyst by centrifugation. The catalyst was then washed three times with 25 ml portions of ethyl alcohol, and the combined solutions were brought to dryness by distillation under reduced pressure at a bath temperature not exceeding 35 C. The residue was then kept in high vacuum until a constant weight was reached. In each case ca. 96% of glycerol-cyclic-phosphodiester was recovered from hydrogenolysis. Thin layer chromatography, with chloroform-methanol-acetic acid 50:48:2 v/v as developing solvent, showed in each case a major spot at R_f 0.21, which was the cyclic-phosphodiester of glycerol, and a very tiny spot at the origin, which was not identified.

After removal of the protective benzyl and phenyl groups, all three *sn*-glycerol-cyclic-phosphodiesters (Fig. 1, structures V-VII) and the racemic mixture (VIII) exhibited a very pleasant aromatic, apple-like aroma. Two of these isomers (Fig. 1, structures V and VI) are optically active enantiomers. The carbon atom in position 2 of the glycerol moiety of V and VI is asymmetric, and the asymmetry of this carbon atom is maintained throughout the synthesis.

TABLE I

Consumption of Periodate in Mole Per Cent by Vicinal Hydroxyl Groups

Glycerol-cyclic-phosphodiester	Periodate consumption, %	Purity of cyclic-phosphodiester, %
V	2.2	97.8
VI	1.3	98.7
VIIa	0.2	99.8
VIIb	0.1	99.9
VIII	12.0	88.0

The analytical values of the glycerol-cyclic-phosphodiesters, after the removal of the protective benzyl and phenyl groups, are listed below.

sn-Glycerol-2,3-cyclic-phosphodiester (V): Specific rotation: $[\alpha]_D^{25}$ C -1.6° ± 0.1°, in ethanol solution, c, 10 (in water, exhibits no rotation). Analysis calculated for $C_3H_7O_5P$ (154): C, 23.39; H, 4.58; P, 20.11. Found: C, 23.41; H, 4.62; P, 20.04.

sn-Glycerol-1,2-cyclic-phosphodiester (VI): Specific rotation: $[\alpha]_D^{25}$ C + 1.6° ± 0.1°, in ethanol solution, c, 10 Found: C, 23.50; H, 4.60; P, 20.09.

sn-Glycerol-1,3-cyclic-phosphodiester (VII): Once the blocking groups, benzyl and phenyl, from IIIa and IIIb were removed, and the acid form of these two compounds was restored, their *trans*- and *cis*-characteristics disappeared and they became identical, i.e., *sn*-glycerol-1,3-cyclic-phosphodiester. Compound VII from IIIa or b had mp 137-138 C, with no melting point depression on mixing. NMR and IR spectra and R_f values of the two preparations were found to be identical. The elementary analyses found: (from a) C, 23.45; H, 4.67; P, 20.03 and (from b) C, 23.95; H, 4.89; P, 19.88.

Racemic glycerol-cyclic-phosphodiester (VIII): Found: C, 23.45; H, 4.67; P, 20.00. At 20 C, of V, VI and VIII, were very viscous liquids. All the isomers were readily soluble at room temperature in water, methanol and ethanol, and insoluble in other common organic solvents.

The purity of glycerol-cyclic-phosphodiester was checked by oxidation with periodic acid for the detection of any free vicinal hydroxyl groups. Results are shown in Table I.

Once the blocking groups from glycerol-cyclic-phosphodiesters were removed, the five-membered ring compounds (V, VI and VIII) were less stable, and slow deterioration of the five-membered ring occurred. To prevent the decomposition of the five-membered ring, isomers V, VI and VIII were converted into their barium-phosphodiester salts.

Preparation of Ba(Glycerol-Cyclic Phosphodiester)$_2$ Compounds of V, VI and VIII

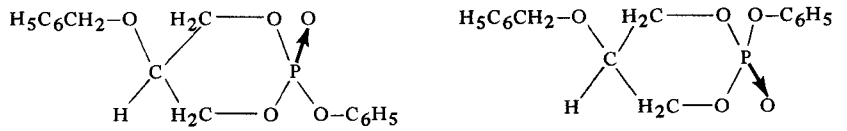
One hundred and fifty grams of Amberlite IRC-50 was suspended in 500 ml of a saturated barium hydroxide solution. The mixture was stirred for 30 min, and the slurry was poured into a glass column, 60 x 4.5 cm, with closed outlet. The outlet was opened and the column tapped occasionally with a rubber hammer to promote uniform settling of the amberlite particles. The excess of barium hydroxide was eluted with distilled water until the effluent was free of barium ions.

The glycerol-cyclic-phosphodiester (7.5 g) was dissolved in 100 ml 80% ethanol or methanol, and the solution was passed through a freshly prepared Amberlite IRC-50 (Ba^{++}) ion exchange column. The column was eluted with ethanol or methanol until the effluent was free of solute, Ba (glycerol-cyclic-phosphodiester)$_2$. The eluate was then concentrated under reduced pressure at a bath temperature of 30-35 C. The concentrated barium salt of cyclic-phosphodiester was freed of the rest of solvent by keeping it in high vacuum until a constant weight was reached. The recovery of Ba (glycerol-cyclic-phosphodiester)$_2$ from the ion exchange column was between 93 and 97% of theory (10-10.5 g).

The following analytical values were found for *Ba (glycerol-cyclic-phosphodiester)*$_2$ of V, VI and VIII: Analysis calculated for $Ba(C_3H_6O_5P)_2$ (443): Ba, 30.97; P, 13.97. Found: V = Ba, 30.93; P, 13.88; VI = Ba, 30.91; P, 13.17 and VIII = 31.05; P, 13.75.

On heating, the barium salt of *sn*-glycerol-2,3-cyclic-phosphodiester (V), softened at 155-160 C and decomposed at 180 C. The isomer 1,2-cyclic-(VI), on heating to 220 C, did not change its appearance. The racemic barium salt (VIII), on heating, behaved similarly to the salt of isomer V. The barium salts of glycerol-cyclic-phosphodiesters were soluble, like free diesters, in water, ethanol, methanol and insoluble in other common organic solvents.

The six-membered ring *sn*-glycerol-1,3-cyclic-phosphodiester (VII) was crystalline and



trans-Form (IIIa) *cis*-Form (IIIb)

FIG. 2. Proposed structures of 2-benzyl-*sn*-glycerol-1,3-cyclic (phenyl)-phosphodiester in *trans*- and *cis*-forms.

quite stable; therefore it was not necessary to convert it into its barium salt.

The success of the synthetic phosphocyclization of the benzyl-*sn*-glycerol with phenylphosphoryl dichloride to produce *sn*-glycerol-cyclic-phosphodiester depends largely on the following experimental conditions: (a) High purity of benzyl-*sn*-glycerol and phenylphosphoryl dichloride is imperative. (b) Solvents pyridine and toluene must be certified spectroanalyzed or equivalent grade and absolutely dry. (c) Phosphocylization of benzyl-*sn*-glycerol with phenylphosphoryl dichloride must be carried out at low temperature, under strictly anhydrous conditions. (d) Reagents must be mixed together simultaneously and in equal portions.

DISCUSSION

As stated in Experimental Procedures, 2-benzyl-*sn*-glycerol-1,3-cyclic(phenyl)-phosphodiester appears in two different forms—one with a high melting point (144 C), considered to be a *trans*-form, and the other with low melting point (71 C), considered to be a *cis*-form. After removal of the benzyl and phenyl blocking groups only one form is found. Identity of the preparations results from loss of configuration due to ionization of the acidic phosphate proton. The phosphorus atom in benzyl-*sn*-glycerol-1,2- and -2,3-cyclic(phenyl)-phosphodiesters is asymmetric. Once the phenyl blocking group is removed the proton is ionizable, and the enantiomeric forms become unresolvable due to ionization and resonance in the ionic species. This postulation is confirmed by the equivalence of preparations of *sn*-glycerol-1,3-cyclic-phosphodiester in acid form from presumed *cis* and *trans* sources.

This study is being extended to the synthesis of acyl-*sn*-glycerol-cyclic-phosphodiesters and -cyclic-phosphosphotriesters. In the latter, the hydroxyl group in the -cyclic-phosphoric acid residue is substituted with choline, ethanolamine or serine, respectively.

Cyclic-phosphodiesters and cyclic-phosphotriesters of glycerol have never been isolated from natural sources. In view of the ubiquitous occurrence of phosphosdiesterases and the lack of appropriate standards, it may have been an impossible task.

Dawson and Clarke (19) have recently revised the structure of myo-inositol phosphate released from phosphatidyl inositol on enzymatic hydrolysis to myo-inositol-1,2-cyclic-phosphodiester.

There is evidence that the cyclic-phosphodiesters of glycerol might serve as intermediates in chemical and enzymic transformation of phospholipids. Preliminary experiments (A. Kuksis, private communication) have shown that the *sn*-glycerol-1,3-cyclic-phosphodiester can serve a substrate of the purified 3′,5′-nucleophosphodiesterase, yielding *sn*-glycerol-3-phosphoric acid ester. Kuksis and O'Doherty (manuscript in preparation) have shown that *sn*-glycerol-1,3- and 2,3-cyclic-phosphodiesters are effective precursors of glycerophospholipids and triglycerides when incubated with microsomes from rat liver and intestinal mucosa in the presence of the diesterase.

Possibly the cyclic-phosphodiesters of glycerol are biochemically important compounds.

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REFERENCES

1. Bailley, O., Bull. Soc. Chim. France 31:838 (1922).
2. Verkade, P.E., J.C. Stoppelenburg and W.D. Cohen, Rec. Trav. Chim. 59:886 (1940).
3. Chargaff, E., J. Biol. Chem. 144:455 (1942).
4. Baer, E., and M. Kates, Ibid. 185:79 (1948).
5. Markham, R., and J.D. Smith, Biochem. J. 52:552 (1952).
6. Baddiley, J., and E.M. Thain, J. Chem. Soc. 3783 (1952).
7. Leloir, L.F., Arch. Biochem. Biophys. 33:186 (1951).
8. Forrest, H.S., and A.R. Todd, J. Chem. Soc. 1950:3295.
9. Ukita, T., N.A. Bates and H.E. Carter, J. Biol. Chem. 216:867 (1955).
10. Brown, D.M., D.I. Magrath and A.R. Todd, J. Chem. Soc. 1952:2708.
11. Maruo, B., and A.A. Benson, J. Biol. Chem. 234:254 (1958).
12. McMurray, W.C., K.P. Strickland, J.F. Berry and R.J. Rossiter, Biochem. J. 66:634 (1957).

13. Khorana, H.G., G.M. Toner, R.S. Wright and G.J. Moffatt, J. Amer. Chem. Soc. 79:430 (1957).
14. Kaufmann, H.P., and N. Förster, Fette Seifen Anstrichm. 62:796 (1960).
15. Sowden, J.C., and H.O.L. Fischer, J. Amer. Chem. Soc. 63:3244 (1941).
16. Hibbert, H., and N.M. Carter, Ibid. 51:1601 (1929).
17. Verkade, P.E., and J.D. van Roon, Rec. Trav. Chim. 61:831 (1942).
18. Gupta, S.C., and F.A. Kummerow, J. Org. Chem. 24:409 (1959).
19. Dawson, R.M.C., and N. Clarke, Biochem. J. 127:113 (1972).

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Changes in Structure of Triglycerides from Maturing Kernels of Corn[1]

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ABSTRACT

Kernels of corn inbred H51 were collected at five intervals after hand pollination. The triglyceride content of the total lipids increased from 8.6% at 10 days after pollination to 78.3% at 60 days. The most active period of triglyceride synthesis occurred from 20 to 45 days after pollination, when the weight of triglycerides per kernel increased from 1.1 to 7.5 mg. Over all the collection periods the percentages of palmitic, linoleic and linolenic acids decreased while oleic acid increased, but from 30 to 60 days after pollination the fatty acid composition of the triglycerides was nearly constant. Stereospecific analysis revealed a general fatty acid pattern for the triglycerides, in which the concentration of the saturated acids was highest in position 1, linoleic acid in 2 and oleic acid in 3. From 20 to 60 days after pollination there was little change in the fatty acid composition at the 1 position, but the largest changes occurred at the 3 position where palmitic and oleic acids decreased 5.1% and 7.3%, respectively, and linoleic acid increased 13.4%. The variations in the molecular species of the triglycerides were determined by silver nitrate thin layer chromatography and were found to be small from 20 to 60 days after pollination, except for an increase in trilinolein from 5.2 to 11.9%. Stereospecific analyses of four major triglyceride species, SMD, M_2D, SD_2, and MD_2, revealed larger changes in fatty acid distribution at individual positions during maturation than were apparent from analyses of the total triglycerides.

INTRODUCTION

The pathway for biosynthesis of triglycerides in plant seeds has not been clearly defined. It has been generally assumed that plants utilize the same pathway as animals, the Kennedy pathway (1). In the final step of this synthesis, preformed 1,2-diacyl-*sn*-glycerol (stereospecific numbering rules approved by IUPAC-IUB Commission on Biochemical Nomenclature [2]) is esterified with fatty acid to form the triglyceride. Other mechanisms have been proposed. Hirayama and Hujii (3) suggested that soybean triglyceride was synthesized from a monoglyceride intermediate, but Privett et al. (4) were unable to confirm this pathway.

Roehm and Privett (5) attempted to clarify the mode of synthesis of soybean triglycerides by studying the changes in triglyceride structure and molecular species composition during maturation of the bean. However, because they used only lipase hydrolysis, they could determine only the fatty acids at the 2 position and a mixture of fatty acids from the 1 and 3 positions. In the present study with corn triglycerides, we have made stereospecific analyses of the triglycerides and their molecular species. This enabled us to determine the changes in the fatty acids at the 1, 2 and 3 positions individually as the corn kernel matured.

MATERIALS AND METHODS

Triglyceride Samples

The corn inbred H51 was grown on the agronomy farm at the University of Illinois, Urbana, during the summer of 1970. Samples were collected at five intervals from 10 to 60 days after hand pollination. The lipids were extracted as described previously (6).

The triglycerides were separated from the other lipid classes by thin layer chromatography (TLC) on plates coated 0.5 mm thick with Silica Gel G (E. Merck, Darmstadt, W. Germany). Approximately 50-60 mg of total lipids were streaked on each plate, and the plates were developed in a nitrogen atmosphere with petroleum ether (60-68 C)-diethyl ether-acetic acid 80:20:1 (7). The triglycerides were located with transmitted light and extracted from the silica gel with diethyl ether-methanol 9:1 (8). Butylated hydroxytoluene (BHT) was added to the isolated triglycerides and in all steps of subsequent procedures to prevent autoxidation.

Stereospecific Analyses

The general technique was based on the method of Brockerhoff (9) and was used as modified in our previous studies (8). The fatty acids at position 1 were identified by analysis

[1]Presented in part at the AOCS Meeting, Ottawa, September 1972.

TABLE I

Accumulation of Lipids in Maturing Kernels of Corn Inbred H51

Days after pollination	Wet wt of 100 kernels, g	Dry wt of 100 kernels, g	Total lipids, % dry wt	Total lipids, wt/100 kernels, mg	Triglycerides, wt% total lipid	Triglycerides, wt/100 kernels, mg
10	6.672	0.835	3.4	28.4	8.6	2.4
20	20.240	5.481	4.8	263.1	43.4	114.2
30	32.873	13.856	5.7	789.8	62.4	492.8
45	30.232	19.932	5.0	996.6	75.6	753.4
60	24.240	20.146	4.6	926.7	78.3	725.6

of the lysophosphatide, those at position 2 by pancreatic lipase hydrolysis, and the fatty acids at position 3 were calculated by difference from the known fatty acid composition of the triglyceride. The results for position 3 were checked by isolating and analyzing the fatty acids of the 2,3-diacyl-phosphatidyl phenols.

Isolation of Triglyceride Species

Silver nitrate TLC was used to fractionate the total triglyceride into its various species according to their degree of unsaturation. The silver nitrate-silica gel TLC plates were prepared as described in a previous publication (10). Two solvent systems were employed. For quantitation of those species containing the trienoic acid, linolenic acid, 0.4 mm thick plates were streaked with 12-15 mg triglyceride and chromatographed with 5% methanol in chloroform. The other species were separated by streaking 5-6.5 mg triglyceride on plates coated 0.3 mm thick and by developing these plates with 0.85-1.5% methanol in chloroform. The methanol in chloroform system was varied according to temperature and humidity conditions. The amount of each triglyceride species was determined from a known weight of methyl heptadecanoate added as an internal standard.

Four major species, SMD, M_2D, SD_2 and MD_2, were isolated from the triglycerides of each harvesting date for stereospecific analysis. (Saturated fatty acids are denoted as S and consist of palmitic acid (16:0) and stearic acid (18:0). Unsaturated fatty acids, oleic (18:1), linoleic (18:2) and linolenic (18:3), are designated as monoene (M), diene (D), and triene (T), respectively.) The petroleum ether solutions of the triglycerides were concentrated to 150 mg/ml in order that 13-20 mg triglycerides could be applied in one pass of the semiautomatic sample applicator (Supelco, Inc., Bellefonte, Pa.) across the TLC plate. These preparative plates were coated with 0.4 mm layers of silver nitrate-silica gel and were developed in 1.3-1.5% methanol in chloroform. The triglyceride fractions were extracted from the silica gel with diethyl ether-methanol 9:1 (10). The weights of the species recovered from the 20 to 60 day samples ranged from 49 to 190 mg. This permitted stereospecific analysis of duplicate samples and, for the more abundant species, triplicate samples. Material was limited in the 10 day species (14-57 mg), allowing duplicate analyses only on the SD_2 fraction. The purities of the molecular species fractions expressed as mean of the five sampling dates ± standard deviation were for SMD 96.9% ± 2.4, M_2D 97.7% ± 1.2, SD_2 98.5% ± 0.9, MD_2 96.6% ± 1.8.

Gas Liquid Chromatography

Fatty acid methyl esters of all the triglyc-

TABLE II

Stereospecific Analyses of Total Triglycerides from Maturing Kernels of Corn Inbred H51[a]

Days after pollination	Compound or position	Fatty acid distribution, mol%				
		16:0	18:0	18:1	18:2	18:3
10	TG[b]	19.5	2.1	15.5	52.4	10.5
	2[c]	1.5	0.6	12.0	73.5	12.4
20	TG	20.9	2.0	32.4	43.3	1.4
	1	32.8	2.9	28.1	35.3	0.9
	2	1.1	0.1	23.2	73.1	2.5
	3	28.9	3.2	45.7	21.4	0.8
30	TG	19.2	1.6	31.0	46.9	1.3
	1	30.8	2.7	26.4	39.4	0.7
	2	1.0	0.1	21.9	75.3	1.7
	3	25.8	2.1	44.8	25.9	1.4
45	TG	18.5	1.4	31.9	46.9	1.3
	1	29.0	3.0	28.7	38.2	1.0
	2	1.1	0.1	24.4	73.2	1.2
	3	25.2	1.2	42.6	29.4	1.6
60	TG	17.5	1.6	31.1	48.8	1.0
	1	27.4	2.7	28.9	40.1	0.8
	2	1.4	0.1	26.0	71.4	1.1
	3	23.8	2.0	38.4	34.8	1.0

[a]Analysis done in triplicate.

[b]TG, triglyceride

[c]Insufficient sample for complete stereospecific analysis.

eride samples were prepared by treatment of the lipids with boron trifluoride-methanol (8). The methyl esters were analyzed in a Hewlett-Packard Model 400 gas chromatograph (Hewlett-Packard, Avondale, Pa.) equipped with a flame ionization detector. The 6 ft x 1/4 in. glass column was packed with 15% diethylene glycol succinate polyester on 80-100 mesh Chromosorb W (AW) (Supelco, Inc., Bellefonte, Pa.). The carrier gas, helium, at 40 psig flowed at 40 ml/min. The column temperature was maintained at 187 C. The percentages of the fatty acids were calculated from peak areas determined by an electronic digital integrator (Hewlett-Packard Model 3370B).

RESULTS

Accumulation of Triglycerides in Maturing Corn Kernels

Data on the accumulation of lipids in maturing kernels of the corn inbred, H51, are shown in Table I. The dry weights of the kernels increased rapidly from 10 to 45 days after pollination, but at 60 days after pollination had leveled off.

The weights of the total lipids per kernel also increased from 10 to 45 days after pollination, but showed a slight decrease at 60 days after pollination. The decrease may be due to sampling error or environmental effects, but for this inbred over 7 years the mean for total lipids at 45 days was 5.1 ± 0.6% of the dry weight and at 60 days 4.8 ± 0.6%.

At 10 days after pollination the kernels contained only a trace of triglycerides. At 20 days the triglycerides as storage lipids were increasing rapidly and accounted for 43% of the total lipids in the kernel. The triglycerides continued to increase both in amount and as percentage of total lipids up to 45 days after pollination. At 60 days the weight of triglycerides per kernel dropped slightly. The major period of triglyceride synthesis was between 20 and 45 days after pollination.

Stereospecific Analyses of Total Triglycerides

The fatty acid compositions of the total triglycerides collected at the different sampling dates are given in Table II. The greatest changes in fatty acid composition occurred from 10 to 20 days after pollination when there was a large gain in the percentage of oleic acid and reductions in the percentages of linoleic and linolenic acids. From 30 to 60 days after pollination the fatty acid composition of the triglycerides was nearly constant.

The fatty acids located at the 1, 2 and 3 positions of the total triglycerides were determined by stereospecific analysis (Table II). The

TABLE III

Proportion of Fatty Acids at Each Position of Total Triglycerides from Maturing Kernels of Corn Inbred H51

Days after pollination	Position	Proportion of fatty acids, %[a]			
		S[b]	M	D	T
20	1	51.8	28.9	27.2	20.8
30		53.6	28.4	28.0	17.6
45		53.7	30.0	27.1	27.2
60		52.4	31.0	27.4	28.2
20	2	1.7	24.0	56.3	59.3
30		1.9	23.5	53.5	44.8
45		2.0	25.5	52.0	31.0
60		2.6	27.8	48.8	37.8
20	3	46.5	47.1	16.5	19.9
30		44.5	48.2	18.4	37.6
45		44.3	44.5	20.9	41.8
60		45.0	41.2	23.8	34.0

[a]Proportion = % fatty acid esterified at that position = % fatty acid in that position x 100/% fatty acid in triglyceride x 3.

[b]Saturated fatty acids (S) consist of palmitic acid (16:0) and stearic acid (18:0). Unsaturated fatty acids, oleic (18:1), linoleic (18:2), and linolenic (18:3), are denoted as monoene (M), diene (D) and triene (T), respectively.

TABLE V

Concentrations of Triglyceride Positional Isomers from Maturing Kernels of Corn Inbred H51

Molecular species	Positional isomer	Days after pollination			
		20	30	45	60
		Total triglyceride, %			
SMD[a]	SMD	0.8	1.1	1.5	1.7
	SDM	10.2	10.3	8.9	8.2
	MSD	0.1	0.2	0.2	0.2
	MDS	5.1	3.8	4.4	4.0
	DSM	0.1	0.1	0.2	0.3
	DMS	2.5	2.2	2.4	3.5
M_2D	DMM	3.1	2.9	3.5	3.4
	MDM	6.6	6.2	7.5	6.9
	MMD	1.2	1.3	2.2	2.5
SD_2	SDD	4.3	5.0	5.7	7.4
	DSD	0.1	0.2	0.2	0.3
	DDS	11.5	9.3	8.3	7.6
MD_2	MDD	4.7	5.0	5.8	6.6
	DMD	0.6	1.0	1.6	2.7
	DDM	15.3	15.0	13.5	11.2

[a]See footnote b to Table III.

TABLE IV

Concentrations of Triglyceride Species from Maturing Kernels of Corn Inbred H51

Triglyceride species	No. of double bonds	Days after pollination				
		10	20	30	45	60
		Total triglyceride, wt%				
S_2M[a]	1	2.5	3.5	2.8	2.4	2.1
SM_2	2	1.8	6.3	7.8	5.6	6.1
S_2D	2	7.7	8.9	8.5	7.2	6.3
M_3	3	1.5	6.5	5.2	5.3	4.9
SMD	3	12.6	18.8	17.7	17.6	17.9
M_2D	4	4.4	10.9	10.4	13.2	12.8
SD_2	4	19.5	15.9	14.5	14.2	15.3
MD_2	5	13.0	20.6	21.0	20.9	20.5
SMT	4	2.1	0.9	0.1	0.2	0.3
M_2T	5	0.6	0.2	0.8	0.6	0.1
D_3	6	14.0	5.2	9.2	11.3	11.9
SDT	5	6.9	0.8	0.6	0.6	0.5
MDT	6	1.4	0.8	0.7	0.5	0.6
D_2T	7	7.3	0.5	0.6	0.5	0.7
ST_2	6	1.9	---	---	---	---
MT_2	7	0.3	---	---	---	---
DT_2	8	2.0	0.1	0.1	---	0.1
T_3	9	0.4	---	---	---	---

[a]See footnote b to Table III.

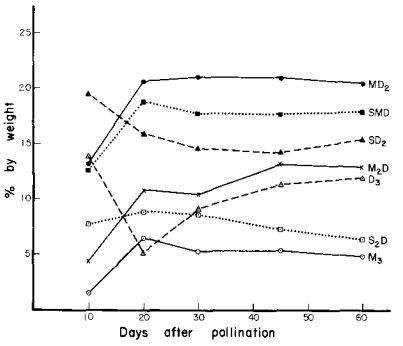

FIG. 1. Changes in major molecular species of triglycerides from corn inbred H51 during maturation of kernels.

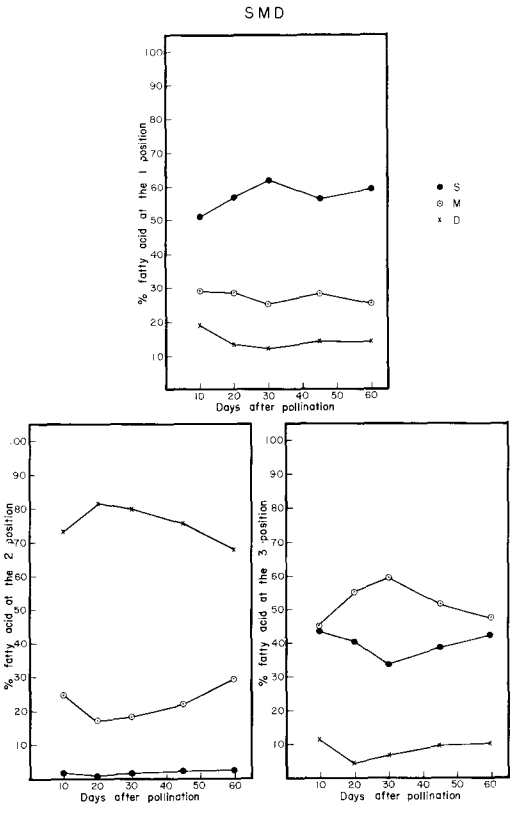

FIG. 2. Changes in fatty acid distribution at three positions of triglyceride molecular species SMD during maturation of corn kernels.

proportion of each fatty acid esterified at a particular position was calculated (Table III). At all sampling dates the proportions of the saturated acids, palmitic and stearic acids, were highest in position 1 and very low in position 2. The proportions of oleic acid, the monoene, were highest in position 3 and slightly lower in position 2 than in 1. Approximately one-half of the diene, linoleic acid, was found at the 2 position. The 1 position ranked second in proportion of linoleic acid and the 3 position third. The values for the triene, linolenic acid, were more erratic because of the small percentages involved, but the proportions of linolenic acid were highest in position 2 and lowest in position 1. This general pattern for the fatty acids in which the saturated acids are predominately esterified at the 1 and 3 positions and linoleic acid at the 2 position agreed with the data previously obtained from corn (8) and many animals (11,12).

Although the overall fatty acid pattern of the triglycerides remained the same, there were changes in the levels of the fatty acids during maturation of the corn kernel. In the 1 position from 20 to 60 days after pollination the percentages of palmitic acid decreased and of linoleic acid increased while those of stearic, oleic and linolenic acids remained relatively constant (Table II). At position 2 over the same period the percentages of oleic acid increased while those of linoleic and linolenic decreased. The largest changes in fatty acid percentages during maturation occurred at the 3 position. Here palmitic and oleic acids showed decreases of 5.1% and 7.3%, respectively, from 20 to 60 days after pollination, and linoleic acid showed a 13.4% increase.

These changes in the distribution of fatty acids at the three positions of the triglycerides occurred at a relatively constant rate from 20 to 60 days after pollination, even though the fatty acid composition of the total triglycerides remained nearly constant from 30 days to 60 days after pollination. Roehm and Privett (5) suggested that in soybean triglycerides the positional distribution of the fatty acids remained virtually unchanged throughout maturation. However with lipase hydrolysis alone they could determine only the variations in the 2 position fatty acids. When the changes at each of the three positions of the triglycerides from corn were compared, the smallest changes occurred at the 2 position.

Triglyceride Species Analysis

Roehm and Privett (5) proposed that extensive changes in relative percentages of the molecular species rather than alterations in distribution of fatty acids within the triglycerides accounted for the changes in the total fatty acid composition of soybean triglycerides during maturation. The extent of the variations of molecular species in corn triglycerides during maturation was determined by quantitating the species with argentation TLC. The results are shown in Table IV. Eighteen different species

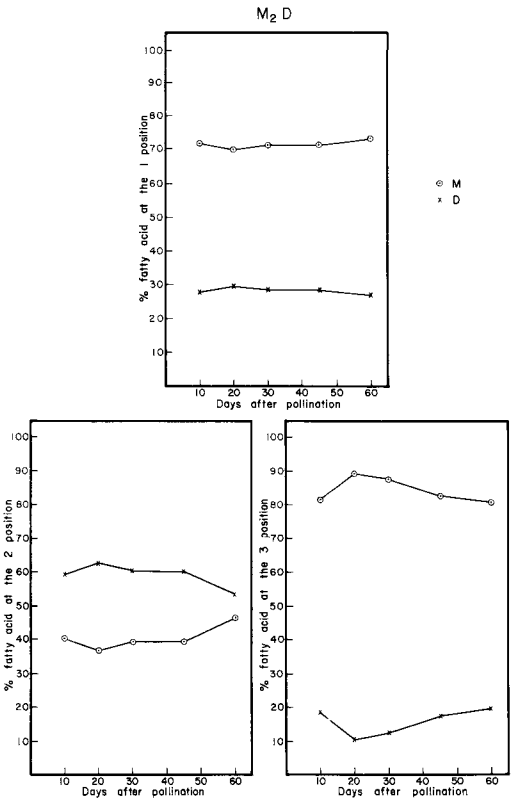

FIG. 3. Changes in fatty acid distribution at three positions of triglyceride molecular species M_2D during maturation of corn kernels.

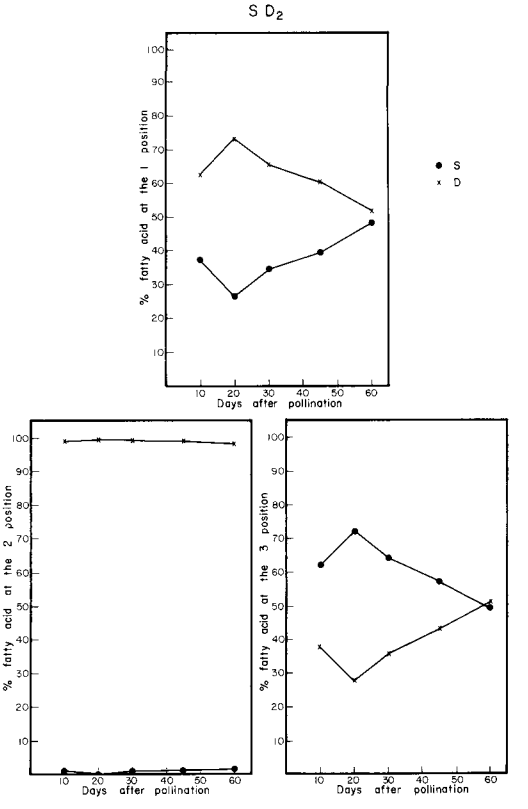

FIG. 4. Changes in fatty acid distribution at three positions of triglyceride molecular species SD_2 during maturation of corn kernels.

were detected at 10 days after pollination, but those species containing linolenic acid either disappeared or became trace components by 20 days after pollination. The changes in the major molecular species are illustrated in Figure 1. During the period of storage triglyceride synthesis from 20 to 60 days after pollination, the species containing the saturated acids remained relatively constant (SM_2) or decreased (S_2M, S_2D, SMD, SD_2, SMT, SDT) reflecting the percentage reduction (3.4%) of saturated acids in the total triglycerides. All of these decreases in the saturated species were small, however, with the largest 2.6% for S_2D. In species containing the monoene, M_3 decreased by 1.6%, M_2D increased by 1.9%, and MD_2 remained nearly constant. Of all the species, D_3 showed the most dramatic change with a gain of 6.7%.

Stereospecific Analyses of Triglyceride Species

The four major species, SMD, M_2D, SD_2 and MD_2, of the corn triglyceride samples were isolated and subjected to stereospecific analyses to determine the variation of fatty acid distribution within each species during maturation of the kernel. For all four species (Figs. 2-5) the fatty acid distribution of the 10 day triglycerides did not fall on the relatively linear lines of change exhibited by the samples collected from 20 to 60 days after pollination. This suggests different structures and functions for the 10 day triglycerides. Therefore we will concentrate on the changes in fatty acid distribution from 20 to 60 days after pollination, the period of storage triglyceride synthesis.

The general fatty acid pattern of SMD (S highest in 1, M in 3 and D in 2) was the same as in the total triglycerides. From 20 to 60 days after pollination there was little change in the fatty acid distribution at position 1 (Fig. 2). In the 2 position S was very low and relatively constant, but M gained 12% and D lost 13.7%. At position 3 there was a lag from 20 to 30 days that did not appear in any of the other molecular species examined, but the general trends were that saturated acids and diene increased, and monoene decreased.

In the M_2D species (Fig. 3) the highest concentration of M was in position 3 and the

lowest in 2, counterbalanced by D highest in 2 and lowest in 3. At the 1 position there was again little change in the fatty acid distribution from 20 to 60 days after pollination. At position 2 there was a 9.3% decrease in D and increase in M, with a nearly equal and opposite 8.7% increase in D and decrease in M at position 3.

S was almost exclusively esterified at the outer 1 and 3 positions of SD_2 (Fig. 4). At the 2 position over 98% was D, and there were essentially no changes at the 2 position during maturation. At the 1 position the diene predominated, and at the 3 position the saturated fatty acids predominated until 60 days after pollination when nearly equal percentages of S and D were found in positions 1 and 3. This final pattern was attained by a large loss (21.3%) of D at position 1 and gain (22.7%) in D at position 3. Reciprocal changes took place in S.

In MD_2 (Fig. 5), M was preferred at position 3 and lowest in 2 with the opposite pattern for D. D was very high in position 2 (ca. 90%). From 20 to 60 days after pollination there were changes in fatty acid distribution at all three positions. The smallest change occurred at position 1, where D showed a decrease of 8.9%. At 2, D decreased 10.1%. The largest change was at the 3 position; here D increased by 20.1%. M exhibited complementary changes at all three positions.

By comparing the changes in fatty acids of the four triglyceride species during maturation some general trends were noted. The smallest changes in fatty acid distribution occurred at the 1 position, except for S_2D. The largest changes were observed in position 3. The percentage of linoleic acid increased at position 3 in all four molecular species, while those of the saturated fatty acids or oleic acid decreased.

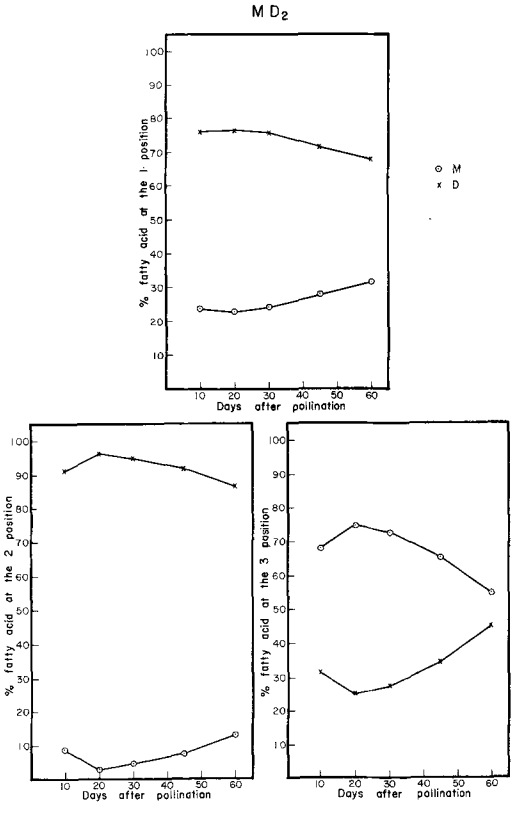

FIG. 5. Changes in fatty acid distribution at three positions of triglyceride molecular species MD_2 during maturation of corn kernels.

DISCUSSION

Stereospecific analyses of the corn total triglycerides have shown a nonrandom distribution of fatty acids among the three positions of the triglyceride molecules, with a general fatty acid pattern for this corn inbred where the saturated fatty acids predominate at position 1, linoleic acid at 2 and oleic acid at 3. Within the general fatty acid pattern, however, during maturation of the corn kernel, changes were observed in fatty acid composition at individual positions. This was not completely due to quantitative changes in the molecular species, because, except for a doubling of the percentage of D_3 from 5.2% to 11.9%, only slight variations occurred in the relative percentages of the molecular species. Stereospecific analysis of individual molecular species revealed large changes in fatty acid distribution, particularly at the 3 position.

In the glycerophosphate pathway of triglyceride synthesis in animals (1), L-α-glycerophosphate is esterified in positions 1 and 2 with fatty acids to form phosphatidic acid. It has not been established whether position 1 or 2 is esterified first. Christie and Moore (13) proposed that the constant proportions of fatty acids found in position 1 of pig triglycerides indicated that this position was acylated first. In corn over the active biosynthetic period of the maturing kernel, position 1 of the total triglycerides had the most constant proportions of fatty acids (Table III). In the molecular species the smallest changes in fatty acid distribution during maturation also were observed at position 1 (Figs. 2-5). The largest changes in fatty acids took place at position 3 of the corn triglycerides. This supports a sequential acylation of positions 1, 2 and 3 as would occur when α-glycerophosphate is acylated to form phosphatidic acid, and the phosphatidic acid is in turn dephosphorylated and

acylated at position 3 to form the triglyceride. Thus our data suggest that the animal pathway for triglyceride synthesis may be operating in corn.

The positional isomers of each molecular species were calculated (12) and are shown in Table V. The percentages of the isomers with M at the 2 position, SMD and DMS, increased in the SMD species, but there was a decrease in the percentages of the isomers with D at the 2 position, SDM and MDS. In the other three species, the percentages of the isomers with D at the 3 position, MDD, DMD, SDD and MMD, were increasing, while those with M or S at the 3 position, DDM, DDS, DMM and MDM, decreased or remained nearly constant. When the weight per kernel of each isomer was calculated, all of the positional isomers showed a gain in milligrams per kernel up to 45 days after pollination, paralleling the increase in total oil. At 60 days after pollination the isomers that had shown a decrease in percentage were also present in lesser amounts in the kernel. Total oil decreased slightly from 45 to 60 days after pollination.

A better understanding of the mechanisms of fatty acid distribution within the triglyceride molecules will have many practical applications. Because fatty acids at the 1 and 3 positions are more susceptible to oxidation (14), the unsaturated fatty acids should be concentrated at the 2 position. The polyunsaturated, essential fatty acids would be conserved if they were located at the 2 position, where they would be absorbed into the intestinal wall as 2-monoglycerides after lipase hydrolysis (15). Recent evidence suggests that, in addition to fatty acid composition, the structure of the triglycerides may influence the atherogenicity of an oil (16). If fatty acid placement can be genetically manipulated, we could produce vegetable oils that are more stable to oxidative rancidity and better nutritionally and physiologically.

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REFERENCES

1. Kennedy, E.P., Fed. Proc. 20:934 (1961).
2. IUPAC-IUB Commission on Biochemical Nomenclature, Biochim. Biophys. Acta 152:1 (1968).
3. Hirayama, O., and K. Hujii, Agr. Biol. Chem. Tokyo 29:1 (1965).
4. Privett, O.S., R.A. Gross, K. Beutel and H. Singh, Presented at the AOCS Meeting, San Francisco, April 1969, Abstr. 28.
5. Roehm, J.N., and O.S. Privett, Lipids 5:353 (1970).
6. Weber, E.J., JAOCS 46:485 (1969).
7. Mangold, H.K., Ibid. 38:708 (1961).
8. Weber, E.J., I.A. de la Roche and D.E. Alexander, Lipids 6:525 (1971).
9. Brockerhoff, H., J. Lipid Res. 8:167 (1967).
10. de la Roche, I.A., E.J. Weber and D.E. Alexander, Lipids 6:537 (1971).
11. Brockerhoff, H., R.J. Hoyle and N. Wolmark, Biochim. Biophys. Acta 116:67 (1966).
12. Slakey, P.M., and W.E.M. Lands, Lipids 3:30 (1968).
13. Christie, W.W., and J.H. Moore, Ibid. 5:921 (1970).
14. Raghuveer, K.G., and E.G. Hammond, JAOCS 44:239 (1967).
15. Raghavan, S.S., and J. Ganguly, Biochem. J. 113:81 (1969).
16. Kritchevsky, D., S.A. Tepper, D. Vesselinovitch and R.W. Wissler, Circulation (Suppl. III) 42:18 (1970).

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Glycerides, Waxes and Sterols in Ovaries of *Ascaris lumbricoides* (Nematoda)

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ABSTRACT

Glycerides (65%), ascaroside esters (33%), waxes (1.5%) and sterols (0.26%) accounted for essentially all the neutral lipids in *A. lumbricoides* ovaries. Nineteen per cent by weight of the triglycerides contained only long chain fatty acids. Nearly all the remaining triglycerides contained 1 mol volatile acid. Mono- and diglycerides, free fatty acids and triglycerides containing 2 mol volatile acids were present in very small amounts. Mole percentages of glyceride volatile acids were α-methylvaleric (70), α-methylbutyric (23), *n*-valeric (ca. 7), and traces of acetic, propionic, isobutyric and *n*-butyric. Mole percentages of the alcoholic component of the waxes were 96% 1-octadecanol, and 4% of its 16, 17 and 19 carbon homologs. The acid components were α-methylbutyric (75 mol%), acetic (16 mol%), propionic and *n*-valeric (each 4 mol%), and traces of *n*-butyric and α-methylvaleric. Sterols (42 wt% cholesterol, 30% cholestanol, 9.5% campestanol, 8.2% stigmastanol, 6.6% β-sitosterol, and 3.8% campesterol) were essentially the same as those found in the whole worm, except that no esterified sterols were present.

INTRODUCTION

Neutral lipids in the ovaries of *Ascaris lumbricoides* are both abundant (1) and complex. Triglycerides predominating in the oocytes are oxidized during embryogenesis (2-4). There are also important amounts of high molecular weight esters of glycosides (ascarosides) that undergo extensive de-esterification following fertilization and are the major components of the ascaroside layer of the egg shell (5,6). Relatively small amounts of cholesterol and saturated sterols are present (7).

It was first surmised (8) and then shown (1) that the "triglyceride" fraction remaining in solution after precipitation of phospholipids with acetone contained esterified volatile acids

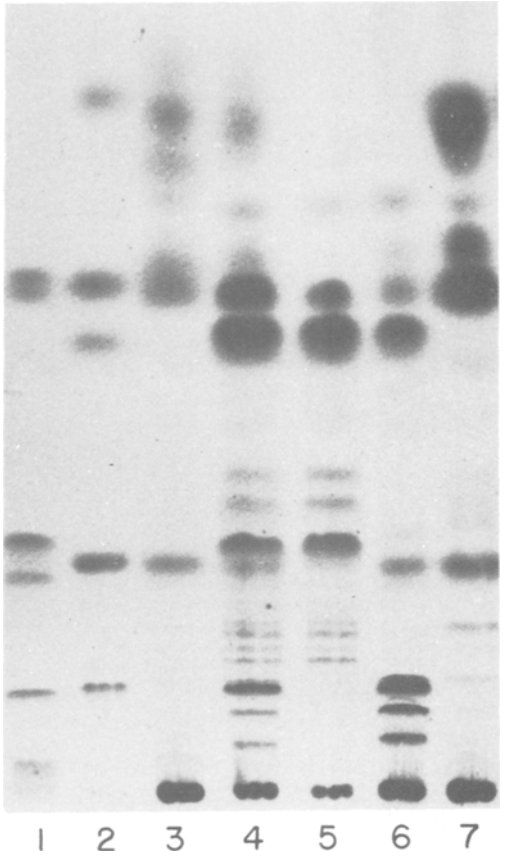

FIG. 1. Gradient-elution, thin layer chromatograms (14) of the neutral lipids of *A. lumbricoides*. Lipids were fractionated on an 80 x 100 mm plate coated with 0.2 mm Adsorbosil-3, with heptane-benzene 1:1/ethyl acetate and detected by sulfuric acid charring. In this system heptane-benzene 1:1 was used to saturate the atmosphere in the tank, and ethyl acetate in the trough served as developing solvent. Constituents are named from top to bottom of the chromatogram. 1. Tristearin, tripalmitin, 1,3-distearin, 1,2-distearin, 1-monostearin and glycerol. 2. Cholesterol palmitate, cholesterol acetate, α-tocopherol, cholesterol and 1-octadecylglyceryl ether. Chromatograms 3-7 are total lipid extracts of males, females, ovaries and fertilized eggs taken from the region of the uterus near the genital pore, and female somatic tissues, respectively. Bands present in the ovaries, but absent in the eggs, are ascaroside esters. Bands near the origin in the eggs, but essentially absent in the ovaries, are free ascarosides (6). Total lipids of males and somatic lipids of females contain several lipids that appear to be ascaroside esters, but are not. Phospholipids are visible at the origin.

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TABLE I

Long Chain Fatty Acids in Two Major Triglyceride Fractions of *Ascaris lumbricoides* Ovaries[a]

Fatty acid	Total long chain acids, wt%	
	Triglycerides containing 3 long chain acids[b]	Triglycerides containing 2 long chain and 1 volatile acid[c]
13:0	0.05	0.03
14:0	0.51	0.31
br15:0	1.24	0.72
15:0	0.08	0.08
16:0	10.2	12.3
16:1	0.21	0.10
17:0	0.64	0.58
17:1	0.12	0.08
18:0	9.6	9.8
18:1	22.9	22.1
18:2	30.7	34.0
18:3(?)	0.47	0.84
20:0	0.36	0.26
20:1(?)	0.16	0.20
20:2	4.42	4.18
20:3	0.79	0.60
20:4	13.9	10.8
20:5(?)	0.34	0.39
22:4	0.75	0.73
(iso)22:4(?)	0.31	0.24
22:5(?)	1.06	0.91
22:6(?)	1.19	0.72

[a]Similar analyses of long chain acids have been reported previously (9,10,15) without reference to the volatile acid constituents (Table II). Identification and quantification was by gas liquid chromatographic analysis on 12% DEGS, 3% DEGS, 3% octadecyl silicone, and comparison with such standard methyl esters as were available. Standards were not available for the C_{20} and C_{22} polyunsaturated acids. Values for the polyunsaturates are less accurate than others. This experiment was the most detailed of three, in which differences in the triglyceride acids were consistent only with respect to br15:0, 16:0, and the C_{20} and C_{22} acids (excluding 21:1, 20:5 and [iso]22:4, which were not always sought).

[b]19% by weight of the total glycerides.

[c]80.5% by weight of the total glycerides.

accounting for ca. 50% of the ester linkages. These originate as fermentation products and consist mostly of acetic, propionic, α-methylbutyric and α-methylvaleric acids (9-11). When it was found that ascaroside esters are exclusively acetates and propionates (6), it seemed reasonable to suppose that most of the esterified α-methylbutyric and α-methylvaleric acids would occur in the triglycerides. This supposition has now been confirmed, and it has also been found that the ovaries contain waxes in which all the acid residues are volatile and all the major volatile acids are represented. The complex mixture of sterols occurring in the whole worm (12) has also been found in the ovaries. None of the sterols were esterified.

MATERIALS AND METHODS

The collection and treatment of biological materials, and general methods for the extraction, saponification, esterification, methanolysis, thin layer chromatography (TLC), and gas liquid chromatography (GLC) of lipids have been described (6). Mono-, di- and tristearins, fatty acid methyl esters, sterols and 1-octadecanol were obtained from Applied Science Labs., State College, Pa. Other standard glycerides were synthesized from the commercial standards as needed. Alcohols other than 1-octadecanol were synthesized by reduction of the corresponding fatty acid methyl esters with lithium aluminum hydride. GLC was used to identify sterols by previously described methods (12), and most fatty acid methyl esters were identified by GLC on columns packed with 12% DEGS, 3% DEGS, 3% QF-1 or 3% bonded octadecyl silicone. Standard methyl esters were available for all fatty acids except those in the C_{20}-C_{24} polyunsaturated group, and the branched chain acids. Identification in the absence of standards was made by comparing the behavior of the unknown methyl esters on several different columns and by reference to standard plots of log retention time vs. carbon number.

TABLE II

Glycerides in Ovaries of *Ascaris lumbricoides*

Glyceride	Total glycerides,[a] %w/w
Triglycerides with 3 long chain fatty acids	19
Triglycerides with 2 long chain fatty acids and 1 volatile acid	
α-Methylvalerate	55
n-Valerate	5.3
α-Methylbutyrate	18.6
n-Butyrate	0.4
Isobutyrate	0.1
Propionate	0.5
Acetate	0.6
Triglycerides with 1 long chain fatty acid and 2 volatile acids	
α-Methylvalerate only	0.2
α-Methylvalerate, α-methylbutyrate	0.1
Diglycerides with 2 long chain fatty acids[b]	0.6
Monoglycerides with 1 long chain fatty acid	Trace

[a]These values were obtained by a combination of methods including fractionation by gradient elution thin layer chromatography (14) in benzene/benzene-ethyl acetate 5:1, gas liquid chromatographic analysis of these fractions for long chain fatty acids and volatile acids, and gas liquid chromatography of the glycerides themselves.

[b]Volatile acids appeared to comprise a small percentage of the diglycerides.

Free volatile acids were isolated from total lipid extracts of adult female *A. lumbricoides* (13) by making the Folch upper phase alkaline, concentrating it at 100 C under nitrogen, extracting the concentrate with diethyl ether, acidifying the aqueous residue and extracting the volatile acids from it with diethyl ether. Three extractions with 6 volumes each of ether removed more than 95% of the acetic acid. The acids were dried as their sodium salts and analyzed by GLC (6). The presence of unsaturated volatile acids was confirmed by GLC of samples dissolved in 0.1 N aqueous HCl before and after treatment with bromine water. The treatment with bromine water resulted in the disappearance of unsaturated acids and was usually accompanied by the appearance of other characteristic peaks.

RESULTS

Thin Layer Chromatograms of Neutral Lipids

Some details of the neutral lipid composition of various tissues of *A. lumbricoides*, as determined by gradient elution chromatography, are apparent in Figure 1.

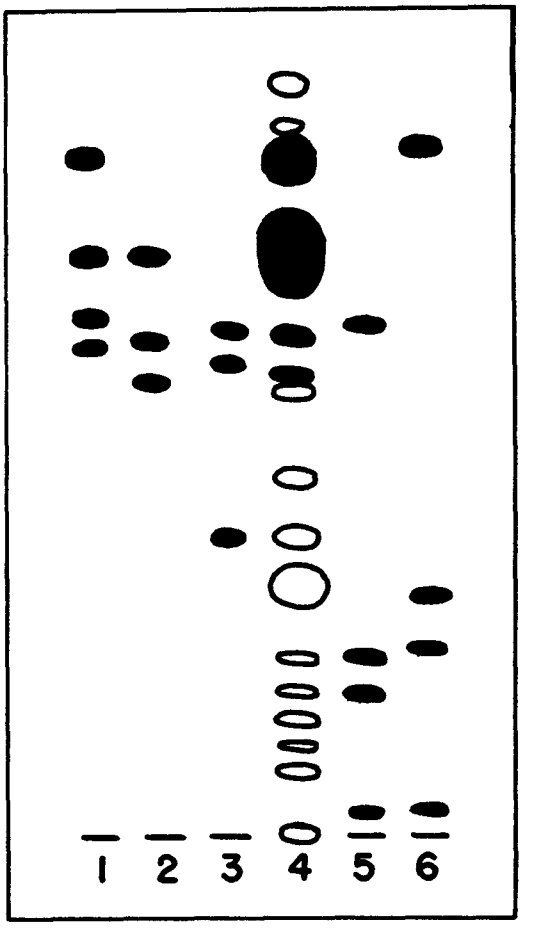

FIG. 2. Thin layer chromatogram of neutral lipids of *Ascaris lumbricoides* ovaries. A 0.2 mm thickness of Adsorbosil-3 on an 80 x 100 mm plate was developed in a gradient elution system (Fig. 1) with benzene/benzene-ethyl acetate 5:1 and charred with H_2SO_4. Fractions are identified from top to bottom of the plate as follows, the nonglyceride bands being depicted only in outline. 1. Standard tristearin, and the caproate, propionate and acetate esters of 1,3-distearin. 2. Caproate, propionate and acetate esters of 1-monostearin. 4. Ovarian lipids. The two faint uppermost bands contained waxes. Below these are four triglyceride bands, the uppermost containing only higher fatty acids and the other containing 1 or 2 mol volatile acids. Most of the remaining bands were ascaroside esters. 5. Bands obtained after brief treatment of 1-monostearin with caproic aanhydride. 6. Tristearin, 1,3-distearin, 1,2-distearin and 1-monostearin.

Glycerides

Fractionation of ovarian lipids by TLC in hexane-diethyl ether-acetic acid gave one large triglyceride band (11). This was resolved into four bands when the lipids were chromatographed in a gradient elution system consisting of benzene/benzene-ethyl acetate 5:1 (Fig. 2).

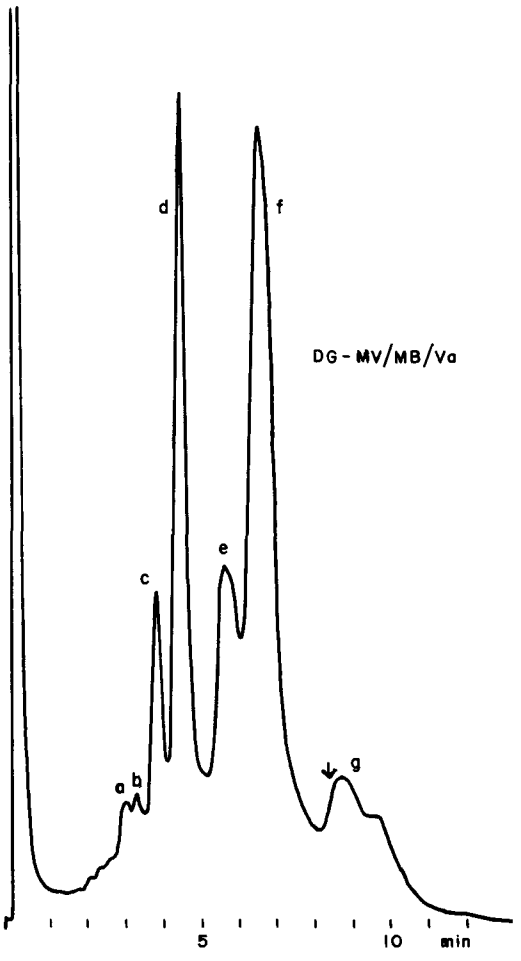

FIG. 3. Gas liquid chromatogram of volatile acid-containing triglycerides found in largest thin layer chromatographic band of *A. lumbricoides* ovaries (Fig. 2). A 1.2 m column, 6 mm ID, was packed with 3% octadecyl silicone on 80-100 mesh Chromosorb-W. Temperature, 300 C; flow rate, 72 ml N_2/min. The following identifications are based on gas liquid chromatographic analysis of the partially resolved triglycerides containing a single 5 or 6 carbon acid (see text) and on a detailed analysis of the long chain fatty acids. Numbers refer to probable chain lengths of constituent acids: (a) 16, 16, 4 and 14, 18, 5; (b) 16, 16, 6 and 14, 18, 6; (c) 16, 18, 5; (d) 16, 18, 6; (e) 18, 18, 5; (f) 18, 18, 6; and (g) 18, 20, 6. The caproate ester of 1,2-distearin (18,18,6) chromatographed at the arrow. The naturally occurring higher fatty acids in ascaris are predominantly unsaturated, while the volatile acids are mainly branched. The retention time of their glycerides on this nonpolar column was therefore less than that of the standard triglycerides.

The position of the uppermost (and second largest) band corresponded to a tristearin standard. It contained no esterified volatile acids, and its long chain fatty acids (Table I) were similar in nature and amount to those described by others (9,10,15).

Triglycerides in the largest band contained 1 mol volatile acids (*n*-valeric, α-methylbutyric or α-methylvaleric) and 2 mol long chain acids (Table II). Long chain acids differed from those of the uppermost TLC band in that there was slightly more palmitic acid, and somewhat less branched chain C_{15} and unsaturated C_{20} and C_{22} acids (Table I). Although the amounts of esterified *n*-valeric acid in different extracts varied from 15 to 29 wt% of the total 5 carbon acids, the ratio of acids with 5 carbons in the longest chain to those with 4 was fairly constant within the range 2.9-3.1. In worms maintained in warm saline in the laboratory for 1-2 days, the ovarian triglycerides also contained small amounts of α-methylcaproic acid and unsaturated 5 and 6 carbon acids of the 2-methyl-*trans*-2-ene series. These were not present in freshly collected worms or in worms kept at 0 C prior to dissection.

The free acid pool of the whole worms, derived mainly from the hemolymph, presented both similarities and contrasts with the esterified volatile acids. Adult females analyzed 0.5 hr after the death of the host contained acetic (14 mol%), propionic (7.5%), isobutyric (0.7%), *n*-butyric (1.0%), α-methylbutyric (53%), *n*-valeric (1.9%), tiglic (2-methyl-*trans*-2-butenoic, 0.1%) and α-methylvaleric (22%) acids. Some samples contained either an acid with the properties of crotonic (*trans*-2-butenoic) acid (0.3%) or an unidentified saturated acid exhibiting a somewhat longer retention time on GLC columns. When ascaris was maintained in vitro for 20-50 hr *n*-butyric, *n*-valeric, crotonic and tiglic acids increased at the expense of acetic, propionic and isobutyric acids. Also appearing in vitro were acids tentatively identified as 2-methyl-*trans*-2-pentenoic, α-methylcaproic, and occasional traces of *n*-caproic. Some of these results were also reported by Harpur and Leigh-Browne (16).

Two minor bands were observed below the largest triglyceride band (Fig. 2). The upper band cochromatographed with the propionate derivative of 1,2-distearin, but not with that of 1,3-distearin. As it migrated slightly below the dicaproate of 1-monostearin, it may also correspond to the dicaproate of 2-monostearin. Similarly, the lower minor band corresponded to both 1,2-distearin acetate and the mixed caproate, valerate of 2-monostearin. Both minor bands gave volatile acids appropriate for these suggested mixtures, and because of the large difference in molecular weight between mono- and diglyceride derivatives, the two components of each band were easily separated and quantified by GLC.

A gas liquid chromatogram of the largest

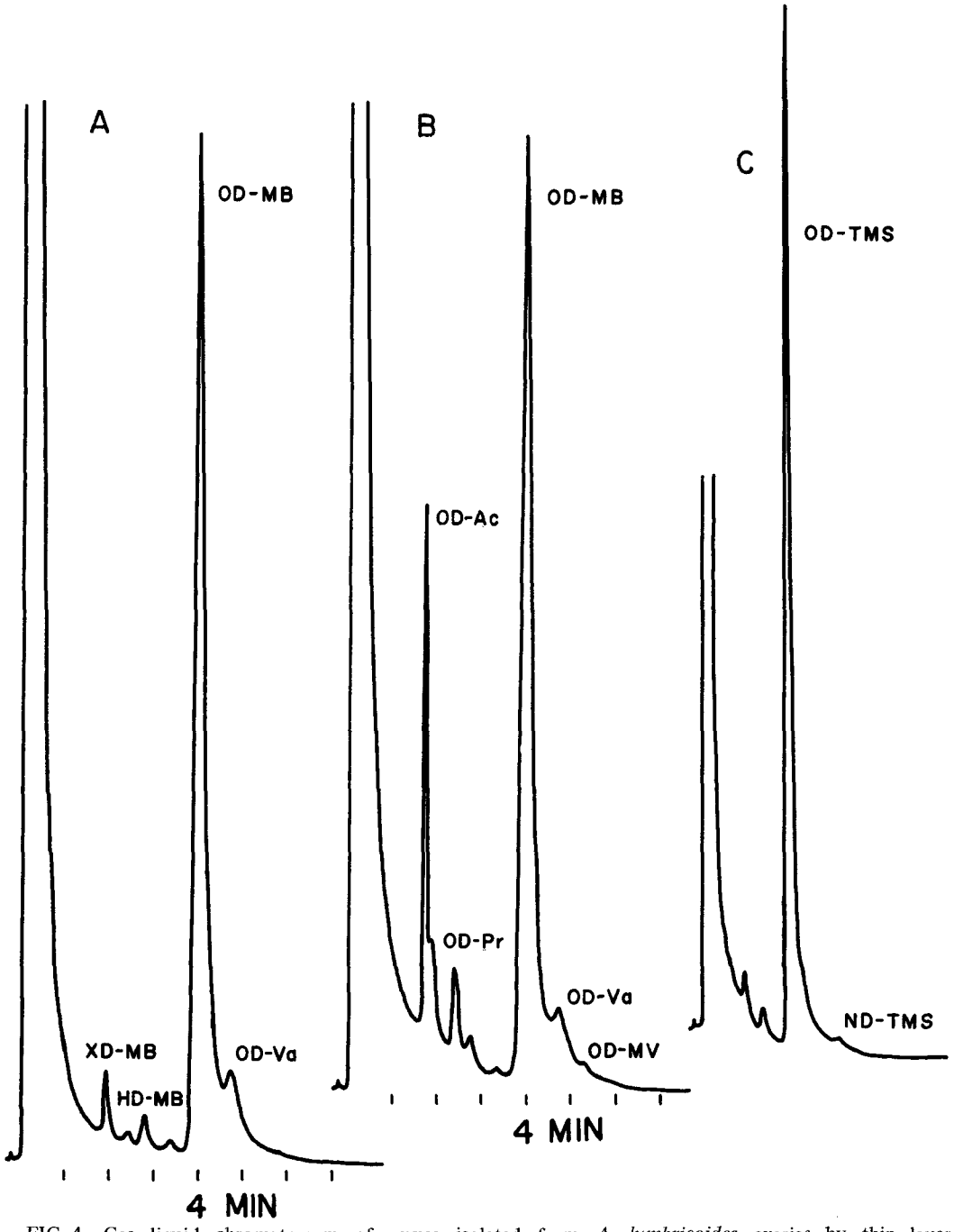

FIG. 4. Gas liquid chromatogram of waxes isolated from *A. lumbricoides* ovaries by thin layer chromatography. A 1.2 m column, 6 mm ID, was packed with 3% octadecyl silicone on 80-100 mesh Chromosorb-W. Temperature, 200 C; gas flow rate, 50 ml N_2/min. (A) Chromatogram of the larger band (Fig. 2) containing only C_5 volatile acids. Major peaks are 1-octadecyl α-methylbutyrate (OD-MB) and *n*-valerate (OD-Va). Minor peaks following hexa- and heptadecyl α-methylbutyrate (XD-MB and HD-MB) are hexadecyl *n*-valerate and heptadecyl *n*-valerate, respectively. (B) Chromatogram of the total wax fraction, in which additional peaks were identified as octadecyl esters of acetate (Ac), propionate (Pr), and α-methylvalerate (MV). (C) Alcohols obtained by saponification of total waxes were chromatographed as their trimethylsilyl (TMS) derivatives. Temperature, 190 C; flow rate, 58 ml N_2/min. The two small peaks preceding octadecyl-TMS (OD-TMS) are XD-TMS and HD-TMS, and the following peak is nonadecyl TMS (ND-TMS).

TABLE III

Waxes in Ovaries of *Ascaris lumbricoides*[a]

Alcohol residue	Acid residue						Total alcohol, mol%
	Acetate	Propionate	Butyrate	α-Methyl-butyrate	*n*-Valerate	α-Methyl-valerate	
	Total wax, wt%						
Hexadecyl	Trace	Trace	—	1.67	0.30	—	1.6
Heptadecyl	0.13	0.03	—	1.59	0.37	—	1.8
Octadecyl	14.0	3.7	0.39	73	3.53	0.58	96
Nonadecyl	0.18	0.05	—	0.20	0.06	—	0.6
Total acid, mol%	15.9	4.0	0.4	75.0	4.2	0.5	

[a]The waxes were fractionated by preparative thin layer chromatography of total lipids in benzene. Both intact and saponified isolates were further fractionated by gas liquid chromatography. All alcohol residues appeared to be saturated, primary and unbranched. Mole per cent total acids and alcohols was calculated from the ester composition of the waxes.

triglyceride band is shown in Figure 3.

Waxes

Jezyk and Fairbairn (3,11) noted the presence of an ovarian lipid that migrated ahead of the triglycerides on TLC plates. Development of the plates with benzene fractionated this material into three bands, among which the uppermost was most abundant. Saponification of the lipid eluted from this major TLC band produced α-methylbutyric and *n*-valeric acids and an alcohol cochromatographing with 1-octadecanol on TLC plates. Most, but not all, of this alcohol also cochromatographed with 1-octadecanol on QF-1 and DEGS columns. Acetates and trimethylsilyl ethers prepared from it were fractionated on octadecyl silicone columns. The fractions corresponded to authentic derivatives prepared from hexa-, hepta-, octa- and nonadecanol. When the intact lipids in the major TLC band were chromatographed on 3% octadecyl silicone columns, two main peaks were observed with retention times corresponding to 1-octadecyl α-methylbutyrate and 1-octadecyl *n*-valerate. Small amounts of hexa- and heptadecyl α-methylbutyrates and *n*-valerates were also present (Fig. 4).

The two minor TLC bands cochromatographed on TLC plates with 1-octadecyl acetate and propionate, respectively. These identifications were confirmed by GLC and by saponification, which yielded acetate, propionate, 1-octadecanol and minor amounts of the other alcohols described in Table III.

Silica gel scraped from the TLC plates just above the major band contained a small amount of 1-octadecyl-α-methylvalerate. Esters of higher acids, whose mobility on the plate would be even greater, were not detected, nor were free fatty alcohols. A complete analysis of the ovarian waxes is presented in Table III.

Sterols

Sterol esters were not present in the ovaries, although they are known to occur in somatic tissues (17).

The weight per cent composition of the free sterols was as follows: cholesterol, 42; cholestanol, 30; campesterol, 3.8; campestanol, 9.5; β-sitosterol, 6.6; and stigmastanol, 8.2. In this series each stanol is a reduction product of the immediately preceding sterol. The values are similar to those previously reported for the whole worm (12,17), although the proportion of stanols is somewhat higher.

Gross Composition of Ovarian Lipids

Total lipids constituted ca. 8% of the fresh weight of the ovaries. Nine per cent of the lipids were phospholipids, in agreement with earlier analyses using a different method (1). The remainder consisted of four classes of neutral lipids (Table IV) whose relative amounts were determined by GLC analysis of the respective classes, as described in the present or a previous (6) report. Other methods such as gravimetry and volatile acid analysis gave similar results. About 98% of the neutral lipids consisted of glycerides and ascaroside esters, the remainder being waxes and sterols.

DISCUSSION

Our results are of interest in terms of the production of volatile acids by *A. lumbricoides* and the distribution of these acids in ovarian triglycerides, ascaroside esters and waxes. Sterols, which essentially complete the list of neutral lipids, are not esterified, and phospholipids do not contain volatile acid (1).

Volatile acids are the main end products of energy metabolism in *A. lumbricoides*. We have confirmed the observation of Harpur and Leigh-

Browne (16) that free volatile acids in freshly obtained parasites are almost completely saturated, but that after *in vitro* maintenance the amounts of unsaturated acids increase.

Most of these fermentation acids are excreted, but in the ovary they are esterified even though the actual production of volatile acids in that organ is in doubt (10). In earlier work, 2-6 carbon volatile acids isolated from the "triglyceride fraction" of the reproductive organs were estimated to constitute 50% of the total ester linkages (1). This triglyceride fraction is now known to include an abundance of ascaroside esters in which only acetyl and propionyl groups occur (6), as well as a small amount of volatile acid-containing waxes. In the present experiments, volatile acids constituted 53 mol% and 16 wt% of the total esters of ovarian neutral lipids. Twenty-eight per cent of the ester groups in the triglycerides were formed from volatile acids.

Our results show in a striking way the high degree of biological specificity that controls the composition of the various ovarian esters. Long chain acids are not present in ascaroside esters, nor are volatile acids other than acetic and propionic. Other aspects of specificity in ascaroside esters have already been discussed (6). All waxes hitherto described seem to consist of long chain alcohols esterified with long chain fatty acids, whereas the ovarian waxes of ascaris contain only volatile acids. Towards these, the organism is not highly selective except with respect to α-methylvaleric acid, which is almost excluded from esterification. Sterols are not esterified at all, and the 5 and 6 carbon volatile acids are overwhelmingly prevalent in the triglycerides. In addition, few triglyceride molecules possess more than one volatile acid.

Although nothing is yet known concerning the biosynthesis of all these highly specific esters, two possibilities that are not mutually exclusive may be mentioned. First, intracellular compartmentalization of substrates and appropriate enzymes may occur. It is known, for example, that much (18) or all (Tarr and Fairbairn, unpublished results) of the ascaroside esters are sequestered in the refringent granules of the oocytes. Whether they are also synthesized in these granules has not been determined. Second, specificity among the glyceride kinases may be well developed. If it is assumed that the pool of free volatile acids in the ovaries is essentially the same as that in the whole worm, it is apparent that in triglyceride synthesis (Table I) *n*-butyric and *n*-valeric acids are selectively esterified relative to their branched chain isomers, so that the longer chain volatile acids in either a homologous or isomeric series are favored. Thus the running chain length of an acid seems to be important in the determination of specificity. Absolute specificity in the kinases seems unlikely, as it was observed that an unnatural 7 carbon acid and two unsaturated volatile acids produced during maintenance of ascaris in vitro, but not present in freshly collected worms, were incorporated into the triglycerides.

TABLE IV

Neutral Lipids of *Ascaris lumbricoides* Ovaries[a]

Neutral lipid	Total neutral lipids, wt%	Unsaponifiables, wt%
Glycerides	65	
Ascaroside esters	33	95
Waxes	1.5	4.5
Sterols	0.26	0.8

[a]The four neutral lipids and the phospholipids comprised at least 99% of total ovarian lipids. Calculations were based on analysis of thin layer chromatographic fractions by gas liquid chromatography and gravimetry. For analysis of ascaroside esters, see Tarr and Fairbairn (6). Phospholipids accounted for 9% of total lipids, analyses being based on fatty acid and aldehyde analysis of material remaining at the origin in neutral lipid solvent systems. The only unidentified neutral lipids were observed in the unsaponifiable fraction. Two of these chromatographed on thin layer plates like a diol and an alkenyl ether. They were not observed by gas liquid chromatography. The third comprised ca. 1% of the monol/sterol fraction. It appeared as a single gas liquid chromatographic peak with a volatility intermediate between octadecanol and cholesterol.

Ascaroside esters are deposited as free ascarosides in the shell of the fertilized egg, and it has been suggested (6) that the acetate thus liberated is used in the synthesis of chitin, which is also an important constituent of the shell. No function can be assigned at present to the ovarian waxes, at least some of which are still present at the time of embryonic cleavage. Long chain fatty acids in the triglycerides are known to be oxidized during embryogenesis (2,4), and the triglyceride volatile acids decrease markedly in early stages of embryogenesis (3). The function of the volatile acids can scarcely be interpreted in terms of caloric value when compared with long chain fatty acids, and therefore remains obscure. A calculation based on in vivo egg production (19), egg weight and per cent lipid (20), secretion of volatile acids (21) and the lipid composition reported here indicates that some 10% of the total volatile acids produced as a result of the energy metabolism of the adult female ascaris may be converted to ovarian esters. If the calculation is limited to acetate and α-methylvalerate, this value approaches 20%.

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REFERENCES

1. Fairbairn, D., Can. J. Biochem. Physiol. 33:31 (1955).
2. Passey, R.F., and D. Fairbairn, Ibid. 35:511 (1957).
3. Jezyk, P.F., and D. Fairbairn, J. Parasit. 55:477 (1969).
4. Ward, C.W., and D. Fairbairn, Devel. Biol. 22:366 (1970).
5. Jezyk, P.F., and D. Fairbairn, Comp. Biochem. Physiol. 23:707 (1967).
6. Tarr, G.E., and D. Fairbairn, Lipids 8:7 (1973).
7. Fairbairn, D., and R.N. Jones, Can. J. Chem. 34:182 (1956).
8. Flury, F., Arch. Exp. Pathol. Pharmacol. 67:275 (1912).
9. Beames, C.G., Jr., Exp. Parasit. 16:291 (1965).
10. Saz, H.J., and O.L. Lescure, Comp. Biochem. Physiol. 18:845 (1966).
11. Jezyk, P.F., and D. Fairbairn, Ibid. 23:691 (1967).
12. Barrett, J., G.D. Cain and D. Fairbairn, J. Parasit. 56:1004 (1970).
13. Folch, J., M. Lees and G.H. Sloane-Stanley, J. Biol. Chem. 226:497 (1957).
14. Tarr, G.E., J. Chromatogr. 52:357 (1970).
15. Jezyk, P.F., Can. J. Biochem. 46:1167 (1968).
16. Harpur, R.P., and G. Leigh-Browne, Exp. Parasit. 29:208 (1971).
17. Cole, R.J., and L.R. Krusberg, Comp. Biochem. Physiol. 21:109 (1967).
18. Foor, W.E., J. Parasit. 53:1245 (1967).
19. Kelley, G.W., and L.T. Smith, Ibid. 42:587 (1956).
20. Fairbairn, D., Can. J. Biochem. Physiol. 33:122 (1955).
21. Harpur, R.P., Comp. Biochem. Physiol. 28:865 (1969).

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Lipid Composition of *Chaetomium cochliodes:* Effect of Media[1]

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ABSTRACT

The fatty acid and lipid compositions of *Chaetomium cochliodes* grown on three different media are compared. Although the growth media effects dramatic changes in the antibiotic production, only minor alterations in the lipid patterns are observed. γ-Linolenic acid was identified for the first time from an ascomycete, and the phylogenetic consequences of this are discussed. In addition, ergosterol was shown to be the major sterol component (95%), the minor sterol being identified as ergosta-5,7,9(11),22-tetraen-3β-ol.

INTRODUCTION

The lipid composition of *Chaetomium cochliodies* Palliser grown on three different media is reported, and γ-linolenic acid has been isolated for the first time from an ascomycete. In addition, both ergosterol and a dehydroergosterol compound have been positively identified. Previous work on *C. cochliodes* (1-5) showed that the production of two intracellular antibiotics was markedly affected by media changes; however only very minor changes were observed in the lipid patterns.

MATERIALS AND METHODS

Organisms

The strain of *C. cochliodes* used is maintained in the collection of this laboratory under the designation HLS-440-1 (6). Though it was originally a single spore isolation, subsequent subcultures were by mass mycelial transfer.

Cultivation Methods

Inocula were prepared in all cases by sterile transfer of a piece of culture on 2% malt agar into 50 ml 2% w/v malt extract (Difco). The cultures were grown at 25 C for 4 days in Erlenmeyer flasks of 125 ml capacity. Each flask was shaken in a horizontal plane at 220 rpm, circling a radius of 1.9 cm. The entire culture was macerated in a Waring blender for 30 sec and the suspension (4 ml) used as inoculum in the production medium (11). Production medium A consisted of 30 g sucrose, sodium nitrate, 1 g dipotassium phosphate, 0.01 g ferrous sulfate heptahydrate, 0.5 g magnesium sulfate heptahydrate, 0.5 g potassium chloride, 3 g calcium carbonate and 5 ml corn steep liquor in water (11). Medium B was the same as medium A but with the corn steep liquor replaced by 2 g Pharmamedia (Traders Oil Mill Co., Fort Worth, Tex.). Medium C was that used by Hauser et al. (7) for the production of the antibiotic chaetocin.

Isolation and Identification Methods

The lipids were extracted from the freeze-dried mycelium with chloroform-methanol 2:1 v/v. The extract was concentrated to dryness, weighed, and the fatty acid methyl esters obtained by transesterification with benzene-methanol-sulfuric acid 10:20:1 for 90 min. The fatty acid methyl esters were fractionated by gas liquid chromatography (GLC) using a 6 ft x 1/8 in. stainless steel column packed with 8%

[1] Issued as NRCC No. 13250.

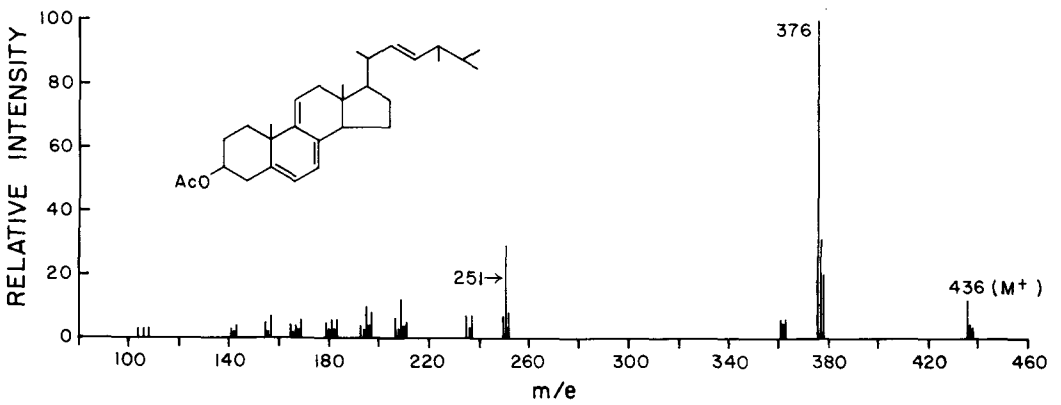

FIG. 1. Mass spectrum of ergosta-5,7,9,(11),22-tetraen-3β-yl acetate.

TABLE I

Lipid Composition[a] of *Chaetomium cochliodes*

Media	Total extract	Total fatty acid methyl ester	Triglyceride	Diglycerides	Sterols	FFA[b]	Chaetomin	Cochliodinol
A	98	32	30.4	2.8	4.7	0.9	4.2	--
B	105	25	30.3	3.3	6.1	1.6	--	--
C	90	22	14.3	0.2	4.5	0.1	1	1.5

[a]Weights are expressed in units of mg/gram freeze-dried mycelium.
[b]FFA = free fatty acid.

diethyleneglycolsuccinate (DEGS) on Chromosorb G support (100-120 mesh) in a Hewlett-Packard 500 gas chromatograph, with a flame ionization detector and helium as the carrier gas (flow rate, 40 ml/min). Relative peak heights were measured using a planimeter. The lipid fractions were separated by thin layer chromatography (TLC) (Merck Silica Gel $HF_{254+366}$), and the unsaturated fatty acids were separated using the above sorbent impregnated with silver nitrate (15% w/w); the sterol acetates were separated on 20% silver nitrate-impregnated plates. Samples for mass spectra were obtained by collecting the appropriate GLC fraction or by isolation of the appropriate bands after argentation TLC. Mass spectra were recorded on a DuPont-CEC 21-110B high resolution mass spectrometer at 70 ev with probe temperature between 0 and 40 C.

RESULTS

The media used were critical with regard to the production of the antibiotics chetomin and cochliodinol. Medium A produced only chetomin and medium C both chetomin and cochliodinol, while medium B gave neither antibiotic. Since the antibiotics were only minor components (ca. 1-5%) of the total chloroform-methanol extract (Table I), it was of interest to examine the lipids present. Analysis of the nonpolar lipids by TLC showed that the lipids produced by *C. cochliodes* were dependent on the media used. Relatively large quantities of triglyceride, 1,3-diglyceride and free sterol were present when media A and B were used; however on medium C no 1,3-diglyceride could be detected. With all three, lesser amounts of 1,2-diglyceride and free fatty acid were also observed. The polar lipids produced several nonfatty acid-containing compounds, and these were not further investigated. However phosphatidyl choline and lysophosphatidyl choline were identified by comparative TLC.

The fatty acid methyl ester compositions of the various lipid fractions are shown in Table II. The fatty acid composition of the mycelia obtained from growth on the three media was similar. The major fatty acids were palmitic, stearic, oleic and linoleic, with lesser amounts of lauric, myristic, palmitoleic and linolenic acids. The fatty acid composition of the diglyceride fraction from medium B and the triglyceride fractions from all three media were similar to the total fatty acid composition. The diglyceride fatty acids from medium C contained slightly less linoleic and more oleic acid, whereas on medium A this fraction was enriched in both of these unsaturated acids. The free fatty acid fractions obtained from mediums A and C

were rich in myristic acid with only relatively minor amounts of unsaturated fatty acids (degree of unsaturation, 0.61 and 0.19, respectively. In all cases the polar lipids contained ca. 20% more linoleic acid than the total and neutral lipid fractions.

Sterols

The free sterol fractions from media A, B and C all contained ergosterol as the major component (⩾95%) with only trace quantities of other sterols present. Acetylation of the sterol fractions and recrystallization of the acetate from ether gave ergosteryl acetate (M^+ = 438, mp 162-163 C, no depression on a mixed melting point with authentic ergosteryl acetate). The mother liquors were further purified by argentation TLC (light petroleum-benzene 5:3), and the less polar band (ca. 5% of the total sterols) gave a molecular ion at m/e 436 indicating a dehydroergosteryl acetate (Fig. 1). This acetate (mp 143-144 C) gave mass, IR and NMR spectral properties identical to those of ergosta-5,7,9(11),22-tetraen-3-β-yl acetate prepared by the method of Zurcher et al. (8).

Identification of Methyl-γ-linolenate

The GLC of the total fatty acid methyl esters isolated from the mycelium grown on each media showed 0.1-0.9% of a methyl ester with an equivalent chain length (ECL) of 20.0 and TLC behavior on silver nitrate-impregnated silica gel plates identical to tht of a triunsaturated ester. Methyl-γ-linolenate has a similar ECL (20.0), and a mixture of the above two esters gave a single peak on GLC. Methyl-γ-linolenate gave an ECL of 20.4 when run under the same conditions. The mass spectra of the isolated ester and the methyl-γ-linolenate were also identical (M^+ at m/e 292).

DISCUSSION

The effect of media on the antibiotic production of *C. cochliodes* is dramatic; however there are only minor alterations of the lipid patterns. Ergosterol has been identified as the major sterol produced on all three media. A minor sterol component was shown to be ergosta-5,7,9(11),22-tetraen-3β-ol, which has recently been identified as a minor sterol component of the fungus *Mucor rouxii* (9).

The fatty acid composition of *Chaetomium globosum* Kunze and *C. thermophile* La Touche has been reported (10), and these closely related species have fatty acids containing a lower degree of unsaturation than *C. cochliodes*. Shaw has also investigated *C. globosum* (11) which, in addition to having a higher degree of unsaturation (1.31) than the isolate

TABLE II
Fatty Acid Composition of Lipids from *Chaetomium cochliodes*

Media	Lipid fraction	Percentage of fatty acids								Degree of unsaturation
		$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{17:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	
A	Total	1.0	1.2	26.8	1.4	---	4.5	12.1	53.0	0.1	1.2
	Triglyceride	0.5	0.9	24.7	1.3	---	7.1	12.0	53.6	---	
	Diglyceride	0.3	0.4	27.5	1.3	---	6.8				
	FFA[a]	47.5	0.5	12.0	1.5		3.5	10.5	24.5	---	
	Polar	1.6	0.3	17.9	1.2	---	0.7	13.1	65.0	0.1	
B	Total	0.3	0.7	14.3	0.9	0.3	2.8	23.2	56.5	0.9	1.4
	Triglyceride	0.8	0.7	13.1	0.5	Trace	4.3	24.4	56.0	0.1	
	Diglyceride	1.1	0.7	15.7	0.7	Trace	2.7	23.5	55.5	0.1	
	FFA	7.9	0.1	11.9	Trace	Trace	3.3	14.6	62.2	Trace	
	Polar	0.9	0.7	12.0	0.5	0.3	0.4	16.3	68.6	0.2	
C	Total	0.1	0.3	18.6	1.1	Trace	2.6	17.1	60.2	0.1	1.4
	Triglyceride	0.6	0.4	20.3	1.1	Trace	4.5	18.1	54.7	Trace	
	Diglyceride	4.5	---	17.5	Trace	---	4.0	22.0	52.0	---	
	FFA	64.5	1.0	14.0	1.0	1.0	6.0	6.5	6.0	---	
	Polar	3.6	0.5	12.2	1.1	0.5	1.0	7.9	73.0	0.1	

[a]FFA = free fatty acid.

above, was shown to contain quantities of α-linolenic acid (7%). Shaw (11) speculated that the presence of γ-linolenic acid is an important phylogenetic marker, distinguishing phycomycetes from ascomycetes, basidiomycetes and Fungi *Imperfecti*. The latter three classes are known to contain α-linolenic acid but were thought to be incapable of producing the γ isomer. The use of these triunsaturated acids as a phylogenetic marker is now somewhat doubtful in the light of the present report, and Sumner (12) has recently shown the presence of γ-linolenic acid in *Dactylaria ampulliforme* (Tubaki) Bhatt & Kendrick (Fungi *Imperfecti*). It is possible, however, that this particular strain of *C. cochliodes* is exceptional, and other strains and related species are currently being investigated.

REFERENCES

1. Taylor, A., in "The Biochemistry of Some Foodborne Microbial Toxins," Edited by R.I. Mateles and G.N. Wogan, M.I.T. Press, Cambridge, 1967, p. 79.
2. Waksman, S.A., and E. Bugie, J. Bacteriol. 48:527 (1944).
3. Brewer, D., W.A. Jerram and A. Taylor, Can. J. Microbiol. 14:861 (1968).
4. Safe, S., and A. Taylor, J. Chem. Soc. Perkin 1. 1972:472.
5. Brewer, D., J.M. Duncan, W.A. Jerram, C.K. Leach, S. Safe, A. Taylor, L.C. Vining, R.M. Archibald, R.G. Stevenson, C.J. Mirocha and C.M. Christenson, Can. J. Microbiol. 18:1129 (1972).
6. Brewer, D., and J.M. Duncan, Can. J. Bot. 46:773 (1968).
7. Hauser, D., H.P. Weber and H.P. Sigg, Helv. Chim. Acta 53:1061 (1970).
8. Zurcher, A., H. Heusser, O. Jeger and P. Geistlich, Ibid. 37:1562 (1954).
9. Atherton, L., J.M. Duncan and S. Safe, Chem. Commun. 1972:882.
10. Mumma, R.O., C.L. Fergus and R.D. Sekura, Lipids 5:10 (1970).
11. Shaw, R., Biochim. Biophys. Acta 98:230 (1965).
12. Sumner, J.L., Can. J. Microbiol. 16:1161 (1970).

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Absorption and Biliary Secretion of Intraperitoneally Injected 3β-Methoxycholest-5-ene-4-^{14}C in the Rat

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ABSTRACT

Following ip injection of 1 mg amounts of methoxycholestene-4-^{14}C into normal intact rats, the amount of label in the whole liver was measured at different time intervals in different animals. These studies indicated that most of the injected compound was absorbed by or before 12 hr. In bile duct-cannulated rats the secretion of label from ip injected methoxycholestene-4-^{14}C or cholesterol-4-^{14}C into bile was usually less during the first 24 hr than during the second 24 hr after injection. Studies on the nature of the labeled compounds in the bile following ip injection of methoxycholestene-4-^{14}C indicate that most of the label is present in metabolites, 47% of the label in cholic acid and 31% in chenodeoxycholic acid.

INTRODUCTION

In addition to the intrinsic biochemical interest in the reactions of conversion of cholesterol to bile acids, there is interest in these reactions as they relate to regulation of blood plasma cholesterol levels via biosynthetic feedback phenomena and by conversion of cholesterol to excretory products. It was primarily with the latter physiological interests in mind that a study of the metabolism of methoxycholestene was undertaken.

The detailed reaction sequences in the conversion of cholesterol to bile acids by mammalian liver have been elucidated in substantial measure (1). The overall reaction sequences may be subdivided into a side-chain cleavage sequence and a second sequence of reactions involving the sterol tetracyclic nucleus. Some interdigitation of side-chain cleavage and sterol nucleus reaction sequences appears to occur with multiple pathways as a result.

One approach to the study of the relationships of the side-chain cleavage to the nucleus alteration reaction sequences would be to either partially or totally block one of the above sequences and then to study remaining reactions that may still be catalyzed. With this hypothesis in mind, the study of the metabolism of 3 β-methoxycholest-5-ene was undertaken, since a stable ether substituent in the 3 position would block important early reactions involving this portion of the sterol nucleus.

After the inception of this work and after some progress had been achieved, a report by Borgström (2) appeared, demonstrating that several orally administered alkoxycholestenes, including methoxycholestene, were quite well absorbed by the rat and reached the thoracic duct lymph intact, without prior ether cleavage. Borgström's study does not permit the conclusion that absorption of methoxycholestene following ip injection would also be without ether cleavage, but it seems likely that this would be the case.

In this communication the rapid appearance of radioactivity into lipid extracts of whole liver, in rats injected intraperitoneally with methoxycholestene-4-^{14}C, is reported. The major proportion of the ^{14}C label appearing in the bile was found present in cholic and chenodeoxycholic acids. The methoxy- substituent of methoxycholestene thus appears not to provide a suitable metabolic block of reactions leading to bile acid formation.

MATERIALS AND METHODS

^{14}C-Labeled Compounds for Injection

Cholesterol-4-^{14}C was obtained from New England Nuclear Corp., Boston, Mass., and from Tracerlab, Waltham, Mass. These were supplied as chromatographically pure products and were used without further purification.

3 β-Methoxycholest-5-ene-4-^{14}C was prepared from cholesterol-4-^{14}C. For a typical preparation, 25 mg carrier cholesterol was added to 250 μCi cholesterol-4-^{14}C (30-50 mCi/mM) in a conical tipped 15 ml tube. Forty milligrams *p*-toluenesulfonyl chloride and 0.1 ml dry pyridine were added to the tube, and the contents mixed until solution in pyridine was complete (3). The reaction tube, after standing overnight in the refrigerator, was placed in an ice bath, and the contents were adjusted to pH 2 (indicator paper) with HCl (2 drops 6 N and 10 drops 0.5 N acid). The mixture was extracted five times with 2 ml portions of diethyl ether. The pooled ether extract was evaporated to dryness under nitrogen. The residue was dissolved in purified methanol, without transfer from the vessel, and heated at reflux for 2 hr (4). The methanol was

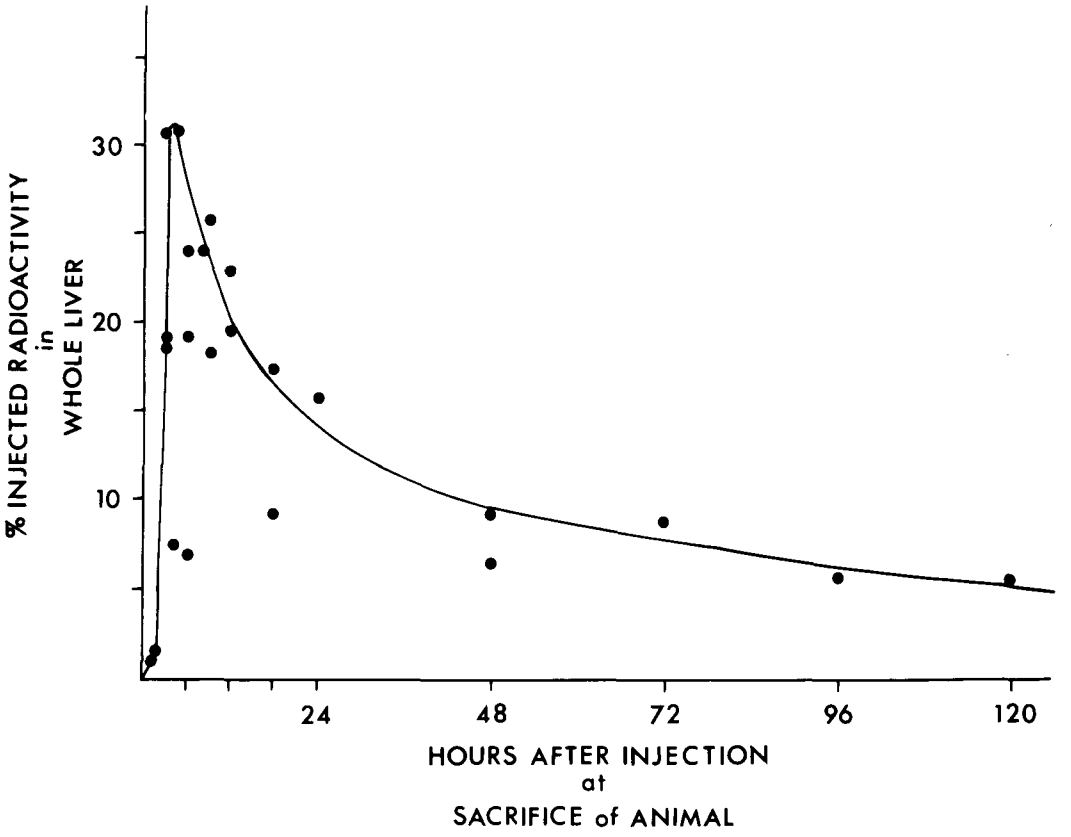

FIG. 1. Recovery of radioactivity in whole liver following ip injections of 3β-methoxycholest-5-ene-4-^{14}C. Measured radioactivity injected into each animal was in range of 7.8-9.5 μCi. Specific activities of methoxycholestene preparations used ranged from 7.9 to 12.4 μCi/mg. Animals used were intact normal males, ranging in weight from 137 to 560 g. All animals were housed, singly caged, in air conditioned retaining room (25±3 C) for at least 1 week prior to experiment and were fed ad libitum. During experiment, animals were kept in air conditioned room at 23±2 C, fed ad libitum, and injected ca. 9:00-10:30 A.M. Each plotted value represents recovery from whole liver of single animal.

evaporated from the reaction vessel under a stream of nitrogen, and the methoxycholestene product isolated and purified by chromatography on a small alumina column. Alumina (2 g) (Camag, chromatographic grade, neutral pH 7.0) was stirred with 0.2 ml water under redistilled petroleum ether (30-60 C boiling), and then transferred to a 10 mm diameter column followed by elutions with petroleum ether. The residue in the reflux vessel was dissolved in 1 ml CCl_4 and placed on the column. Two more 1 ml portions of CCl_4 were used to make the transfer quantitative. The radiomethoxycholestene was then eluted from the column in the first fraction with 40 ml redistilled petroleum ether (30-60 C boiling). The solvent was removed from the radiomethoxycholestene (I) under reduced pressure, and the product was dried in vacuo, weighed, dissolved in isooctane, and transferred quantitatively to a 50 ml volumetric flask. Aliquots of this solution were used for characterization by thin layer chromatography (TLC), radioassay by liquid scintillation counting for specific activity, and subsequently for ip injections. The radiomethoxycholestene (I), was compared by TLC to a nonradioactive sample of methoxycholestene (II) prepared in 6 g quantity by the methods described above (3,4), except that the cholesteryl *p*-toluenesulfonate intermediate was recrystallized twice from ether and the methoxycholestene (II) three times from methanol. The methoxycholestene (II) had a mp of 81-82 C, (uncorrected); lit. value, 84 C, Diels and Blumberg, cited by Stoll (4). TLC was on 250 μ thick plates of Silica Gel G (according to Stahl) with a C_6H_6/CHI_3 1:1 v/v solvent system, R_f methoxycholestene, ca. 0.3. TLC revealed the radiomethoxycholestene (I) to chromatograph as a single spot, free of cholesterol and of the *p*-toluenesulfonate intermediate. The single radiomethoxycholestene (I) spot

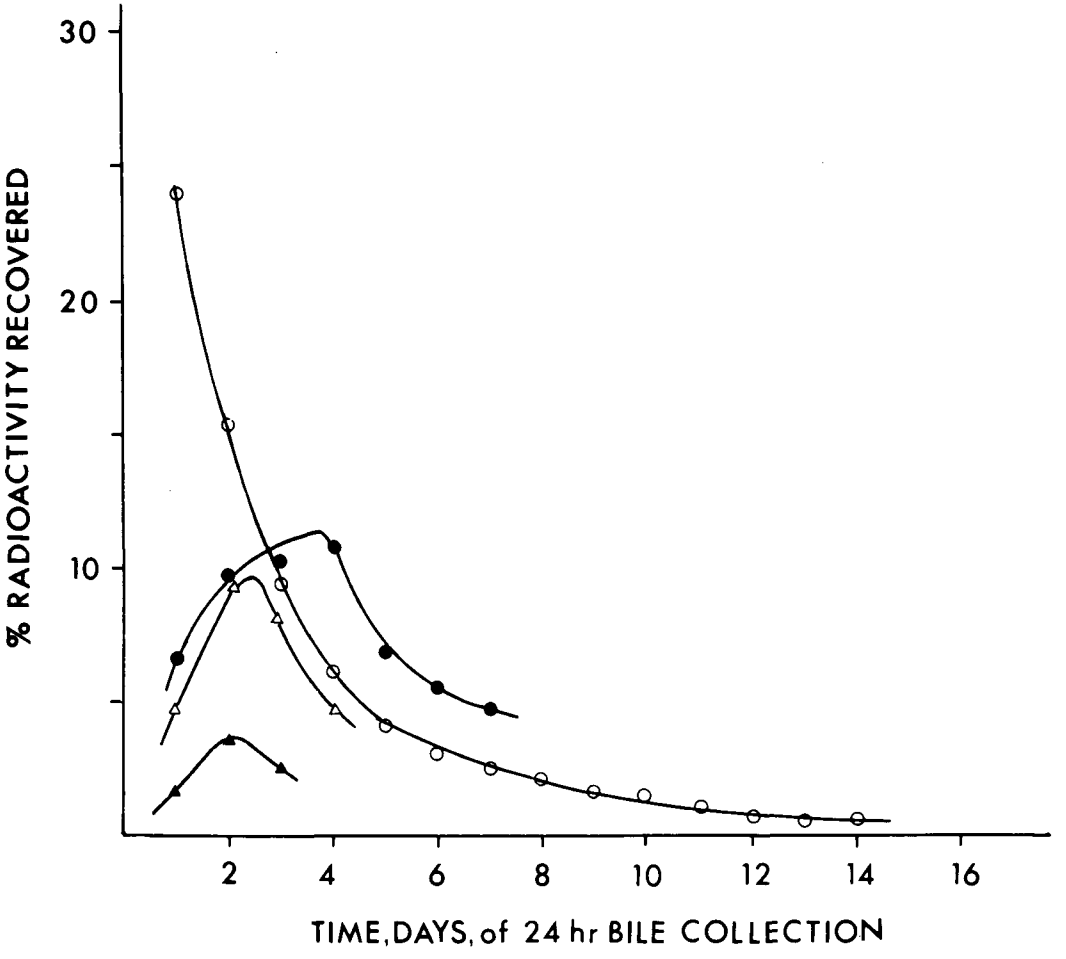

FIG. 2. Recovery of injected radioactivity in bile following ip injection of 3β-methoxycholest-5-ene-4-^{14}C or cholesterol-4-^{14}C in bile duct-cannulated rat. -▲-▲-▲-, experiment 1: 8.4 μCi (specific activity 12.4 μCi/mg) of methoxycholestene injected. -△-△-△-, Experiment 2: 7.6 μCi (specific activity 7.92 μCi/mg) of methoxycholestene injected. -○-○-○-, Experiment 3: 7.4 μCi (specific activity 6.34 μCi/mg) of cholesterol injected. -●-●-●-, Experiment 4: same injection as experiment 3.

had the same R_f value as the authentic methoxycholestene (II) sample. The radiomethoxycholestene (I) thus showed high purity chromatographically. Yields of radiomethoxycholestene (I) (mass and radiochemical) in excess of 90% were readily obtained.

For purposes of comparison, a few studies of secretion of label into bile following ip injection of cholesterol-4-^{14}C were made. Amounts of cholesterol-4-^{14}C or methoxycholestene-4-^{14}C used for the ip injections were ca. 10 μCi/mg. The compounds were injected as suspensions in 0.7 ml of 0.9% saline stabilized with 1 drop of Tween 20 (5).

Experimental Animals

Animals used were adult Sprague Dawley rats. Intraperitoneal injections were made in the morning in ad libitum fed animals.

Bile duct cannulas, PE 10 polyethylene (Clay-Adams), were inserted and secured in the bile duct close to the liver, and the cannulas were led out through the right dorsolateral abdominal wall. These manipulations and completion of the surgical procedures were done with animals under ether anaesthesia. The animals were then placed in Bollman type restraining cages and were fed a pelleted commercial rat food and water ad libitum.

In all cases ip injections were made 24 hours after the insertion of the cannula and establishment of the fistula.

Extraction of Steroids, Metabolites and Lipids from Liver and Bile

For determination of label in the liver, the

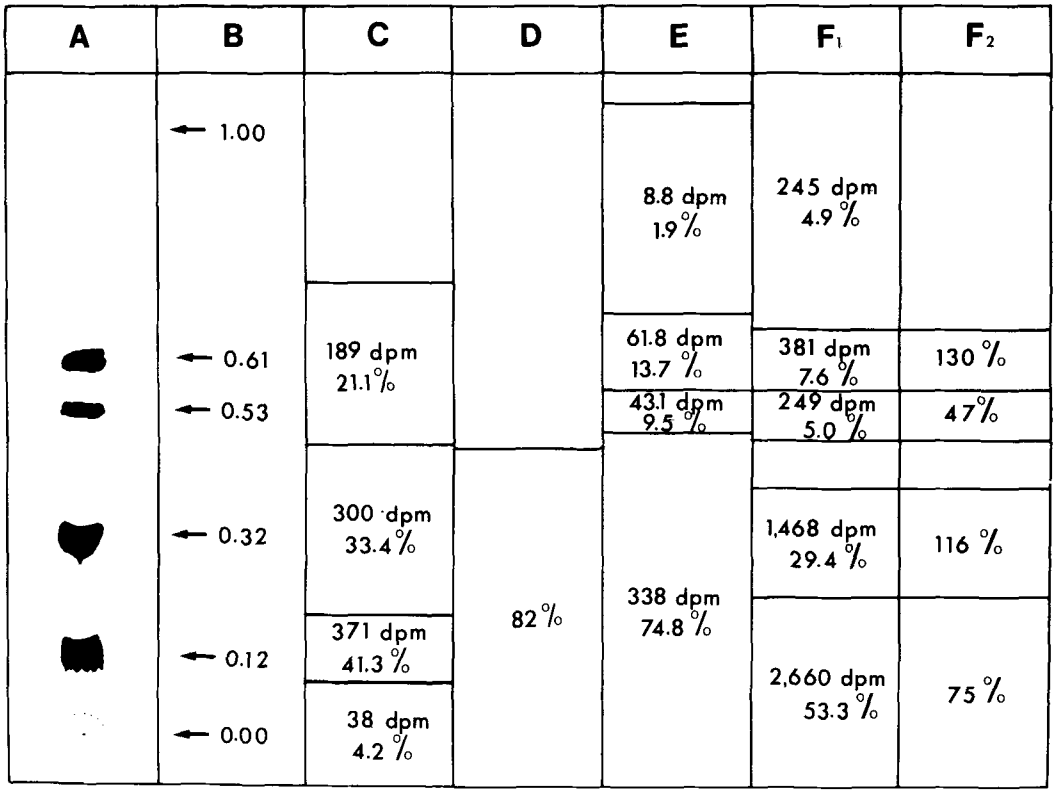

FIG. 3. Methoxycholestene metabolites in bile, studied by thin layer chromatography, experiment 2 (see legend, Fig. 2). Thin layer chromatography on Silica Gel G, 250 μ thick, solvent system toluene-acetic acid-chloroform-water 80:36:20:1 v/v. Diagram is drawn to scale of R_f values of chromatograms. Positions of areas to be scraped off were determined by chromatographing in duplicate in adjacent lanes, then spraying one lane with phosphomolybdic acid spray while protecting adjacent lane with baffle. After color development and spot visualization (sprayed with 5% phosphomolybdic acid in ethanol-diethyl ether 1:1, then heated at 100 C for 3-5 min), areas to be scraped from untreated lane were marked. Panel A: diagram of typical chromatogram of extract of hydrolyzed bile. Panel B: R_f values, methoxycholestene, 0.61; cholesterol, 0.53; chenodeoxycholic acid, 0.32; cholic acid, 0.12. Panel C: distribution of radioactive counts from hydrolyzed bile extract, first 24 hr bile sample after ip injection; dpm and % total counts are metabolites from fraction shown which were bound to Amberlite IRA-400-OH resin, first 24 hr bile sample. Panel E: dpm and % total counts, first 24 hr bile sample. Panel F_1: dpm and % total counts, second 24 hr bile sample. Panel F_2: eluates from F_1 recrystallized three times with nonradioactive carrier compounds; % recovery of dpm in crystallized compounds corrected for losses during crystallization.

rinsed whole livers of decapitated rats were blotted dry and homogenized in ethanol; then each liver was ectracted exhaustively (five extractions at boiling) with ethanol plus concentrated HCl and with chloroform-methanol 2:1 v/v. The five extracts from a single liver were pooled, taken to dryness under reduced pressure, dissolved in successive small portions of $CHCl_3/CH_3OH$ 2:1, filtered into a 100 ml volumetric flask, and made to volume. Aliquots of these lipid solutions were used for radioassay.

The bile samples were extracted with five volumes or more of repurified absolute ethanol, filtered, then brought to standard volume. Aliquots were taken for radioassay.

Radioactive Assay of Liver and Bile Extracts

Aliquots of the extracts, following removal of solvent, were counted at ca. 64% efficiency in a liquid scintillation counter. The counts obtained were corrected for quenching by use of an internal standard of benzoic acid-^{14}C.

Distribution of Label from Injected Methoxycholestene-4-^{14}C in Compounds in Bile

These studies were done on alkali-hydrolyzed bile, to convert the bile acid conjugates to free bile acids. Hydrolysis was done with 7 N NaOH, 3 hr, 121 C at 15 psig. The hydrolyzate was acidified, extracted multistep with diethyl ether, water-washed, evaporated to remove solvent, and the residue made to standard volume

in $CHCl_3/CH_3OH$ 2:1 v/v.

Distribution studies of label in compounds of hydrolyzed bile were based primarily on TLC with several additional techniques as shown in Figure 3.

RESULTS

Absorption of Methoxycholestene-4-^{14}C and Recovery in Liver of Radioactivity Following Injection

The results with individual normal intact rats are given in Figure 1. The percent recoveries plotted are the per cent of injected radioactivity present in the lipid extract of the whole liver and may represent the injected parent compound as well as metabolites. At 1-1.5 hr the amount of radioactivity in the liver is very small, whereas at 3 hr about one-fifth of the injected radioactivity is present in the liver. The amount of radioactivity in the blood was not measured but could be several-fold larger if the distribution is similar to that of cholesterol between blood and liver. From the data it may be estimated that most of the compound is absorbed by 12 hr. Not unexpectedly, there is variation in the amounts of radioactivity in the livers of different animals during the first 24 hr, and subsequently the pattern becomes uniform among animals. We have not made similar absorption studies in bile duct-cannulated rats. The finding that methoxycholest-5-ene is well absorbed in normal rats following ip injection is thus in accord with the findings of Borgström (2).

Biliary Secretion of Label Following Intraperitoneal Injection of 3 β-Methoxycholest-5-ene-4-^{14}C and Cholesterol-4-^{14}C

Data on secretion of label into the bile are presented in Figure 2. The two methoxycholestene-injected animals secreted less label during the same time period than did the two cholesterol-injected animals; however the pattern of secretion of label was similar for both compounds. In three of the four experiments secretion of label into the bile was delayed during the first 24 hr period. Bile flow volumes in all four experiments ranged from ca. 11.0 ml to 20.0 ml per 24 hr, indicating good animal preparations. Cumulative secretion of label following cholesterol injection was 54.6% of the injected radioactivity in 7 days (experiment 4) and 73.2% in 14 days (experiment 3). These values far exceed that reported by Bergström (6) and cited by Berström et al. (7). Berström found that 25% of ip injected cholesterol-4-^{14}C was secreted as bile salt in a 10 day period. Radioactivity from unmetabolized cholesterol-4-^{14}C would account for some of the increased amounts in our studies.

While the secretion of label may be less for methoxycholestene than for cholesterol, the data do establish that label in substantial amount is secreted into the bile when methoxycholestene is injected.

Nature of Metabolites of Methoxycholestene

The data are presented in Figure 3. Panels C, E and F_1 show that 75-83% of the label in the bile was present in compounds more polar than methoxycholestene or cholesterol. Panel D shows that, of the metabolites more polar than methoxycholestene or cholesterol, 82% could be bound to the anion exchange resin Amberlite IRA-400-OH. Panel F_2 shows that recrystallization of eluate fractions from F_1 with nonradioactive carrier compounds provides additional evidence that the principal metabolites are cholic and chenodeoxycholic acids. Panels E, F_1 and F_2 show that radioactivity is also present in both methoxycholestene and cholesterol, with more count in the parent methoxycholestene. The mean value of the ratio of cholic-chenodeoxycholic acids from Panels C and F_1 is 47/31. This may be compared with a ratio of 80/20, cited by Van Belle (8), for metabolism of cholesterol to bile acids in the rat. We have not studied this ratio in the bile of our cholesterol-injected rats.

DISCUSSION

In both of the methoxycholestene-^{14}C and in one of the two cholesterol-^{14}C ip injection studies, secretion of label into the bile during the first 24 hr period was less than during the second 24 hr period. Peric-Golia (9) administered cholesterol-^{14}C iv to bile duct-cannulated rats 48 hr after placement of the cannulas by surgery and also observed a delay of biliary secretion of the label, similar to our own observations. Klauda and Quackenbush (10) have recently observed that cholesterol, administered *per os*, on the first day following the day of thoracic duct cannulation was absorbed to the extent of less than half that on subsequent days. They report that this phenomenon appeared not to be a function of sex, fasting, type of anaesthetic, heparin injection or mode of administration of the test dose. They suggested as a possible explanation a lag in recovery of normal physiological processes following surgery.

The diminished ratio of cholic-chenodeoxycholic acid produced by metabolism of methoxycholestene compared with cholesterol is of some interest. This may be a result of a greater proportion of methoxycholestene than

cholesterol undergoing an initial hydroxylation in the side chain at position 26. With hydroxylation in the side chain, and the resulting inhibition of the 12α-hydroxylase, a greater flux to chenodeoxycholic acid is produced.

ACKNOWLEDGMENTS

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REFERENCES

1. Danielsson, H., and Tchen, T.T., in "Metabolic Pathways," Academic Press, New York, Third Edition, Vol. 2, Edited by D.M. Greenberg, 1968, p. 117.
2. Borgström, B., Proc. Soc. Exp. Biol. Med. 127:1120 (1968).
3. Wallis, E.S., E. Fernholz and F.T. Gephart, J. Amer. Chem. Soc. 59:137 (1937).
4. Stoll, W., Hoppe Sey. Zeit. 207:147 (1932); Ibid. 246:1 (1937).
5. Harold, F.M., M.E. Jayko and I.L. Chaikoff, J. Biol. Chem. 216:439 (1955).
6. Bergström, S., Kungl. Fysiografiska Sällskapets i Lund Förhandlingar 22(16):1 (1952).
7. Bergström, S., H. Danielsson and B. Samuelsson, in "Lipid Metabolism," Edited by K. Bloch, John Wiley and Sons, New York, Inc., 1960, p. 295.
8. Van Belle, H., in "Cholesterol, Bile Acids, and Atherosclerosis," North-Holland Publishing Co., Amsterdam, 1965, p. 98.
9. Peric-Golia, L., Lipids 4:147 (1969).
10. Klauda, H.C., and F.W. Quackenbush, Ibid. 5:142 (1970).

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SHORT COMMUNICATIONS

Vitamin A: Occurrence and Distribution in Fractionated Mucus

ABSTRACT

Vitamin A was found in human and bovine mucus, principally as retinyl esters, and was traced to a fraction containing the mucus globule membrane, which was obtained by differential centrifugation of dissolved mucus. This fraction has not been isolated before.

Vitamin A has long been known to play an essential role in the secretion of mucus, for the drying and metaplasia of mucus membranes are among the earliest signs of vitamin A deficiency (1). The metabolic fate of the vitamin in mucus-secreting cells has never been established, although the possibility that it is converted into an unidentified "active" metabolite before functioning has been the basis of much research. A direct approach to this question through the analysis of mucus for vitamin A has not been taken. Since previous studies (2,3) have shown that lipids are normal components of mucus, analyses were made to determine if vitamin A also occurred in mucus. This investigator found vitamin A in both human and bovine mucus, and traced it to a fraction believed to contain the mucus globule membrane (MGM). This membrane has not been noted in previous analyses of mucus, nor has it been isolated before.

Human nasal and bronchial mucus were obtained from two patients, one having a common cold and ensuing bronchitis and the other having an asthmatic condition that permitted daily collections of 75-100 ml. Care was taken to prevent contamination from food. Bovine cervical mucus was obtained from the slaughterhouse and pooled into lots large enough for analysis. Each lot contained traces of blood. The samples were frozen prior to analysis.

The mucus was dehydrated in methanol which was then extracted with hexane after being diluted with 0.5 volume of water. The mucus residues were homogenized in chloroform-methanol 2:1 v/v and filtered. The filtrate was washed with 0.2 volume of 0.1 NaCl, and the combined extracts were taken to dryness at 40 C under vacuum for weighing. The lipid was taken up in hexane and partitioned against 90% aqueous methanol under nitrogen to concentrate the retinyl esters. The epiphase was analyzed by thin layer chromatography (TLC) on 350 μ layers of Silica Gel G in a solvent system of hexane-diethyl ether-acetic acid 94:6:1 v/v/v. Authentic retinyl palmitate (BDH Chemicals Ltd.) was analyzed in an adjoining channel for comparison of Rf values and fluorescence. A hexane extract of the hypophase was analyzed similarly in a solvent system of hexane-diethyl ether-acetic acid 50:30:1 v/v/v with retinol (BDH Chemicals Ltd.) in an adjoining channel. After development, the plates were examined briefly under 350 nm UV light. The yellow fluorescent band, which corresponded to the appropriate reference compound was scraped from the plate, eluted with

TABLE I

Lipid and Vitamin A Contents of whole mucus

Source of mucus	Mucus wt, g	Lipid wt, mg	Per cent lipid	Retinyl esters		Retinol	
				μg[a]	ppm[b]	μg[a]	ppm[b]
Human nasal	24	71	0.29	0.11	1.6	Trace	---
Human bronchial -1	65	255	0.39	0.6	2.3	0.3	1.1
Human bronchial -2	89	497	0.56	0.8	1.7	0.4	0.9
Human asthmatic	316	152	0.05	0.3	2.0	Trace	---
Bovine cervical -1	43	31	0.072	1.1	35	Trace	---
Bovine cervical -2	89	69	0.078	1.4	20	Trace	---

[a]Representing recoveries of 10%.
[b]Relative to lipid weight.

TABLE II

Lipid Yield from Three Samples of Human Asthmatic Mucus Fractionated by Differential Centrifugation

Sample	Wt, g	Lipid	Lipid, mg			
			Fraction 1,[a] cellular	Fraction 2,[a] MGM	Fraction 3, ppt	Fraction 4, sol
1	135	0.125%	111.2	56.3	1.3	0.5
2	154	0.057%	45.2	41.4	0.6	0.2
3	173	0.067%	58.4	49.4	3.0	2.0
		Pooled:	215.4	147.1		
			No vitamin A	Vitamin A present		

[a]Pooled and tested for vitamin A.

diethyl ether and scanned in a recording UV spectrophotometer (Beckman DB). The solution was then concentrated, rechromatographed on TLC with an adjoining reference compound, and the fluorescent area tested for color response to trifluoroacetic acid (TFA) (4). The amount of vitamin A was calculated from the optical density at 325 nm using a conversion factor of 1600. To test recoveries, a known amount of retinyl palmitate was added to an aqueous solution of extracted mucus residue, used to simulate normal mucus, and analyzed by the above procedures.

Four criteria were thus used to establish the presence of vitamin A: (a) yellow fluorescence under UV light; (b) Rf values of these fluorescent bands being the same as vitamin A reference compounds; (c) a maximum UV absorbance at 325 nm, and (d) blue color response to TFA. The detection of low concentrations of vitamin A in mucus depended upon the analysis of sufficient amounts of lipid. With marginal amounts, the 325 nm maximum was not observed, although the other tests were positive. Only the results that could be quantitated are listed in Table I, but retinyl esters were detected in an equal number of other samples. Carotenoids were invariably present. Retinol was detected in many of the samples, but only two contained enough to be quantitated. Vitamin A occurs in the blood largely as retinol (5), but the traces of blood in the bovine cervical mucus did not raise retinol levels, indicating that this contamination was not significant. Small amounts of retinol are known to result from the hydrolysis of retinyl esters in frozen tissue (6). The concentrations of vitamin A represent recoveries of ca. 10%. Vitamin A is extremely labile, and losses of 90% were found when mixtures of vitamin A and mucus residues were analyzed by the same procedures. A 40% loss occurred during a single TLC analysis.

At this point the vitamin A could have originated from either the cells which continually exfoliate from the epithelial mucosa or from the mucus globules. The latter are enclosed during secretion in a membrane derived from the Golgi complex (7). Thus there appear to be three possible sources of lipid from mucus: cells, the MGM and the globule contents. Previous studies of mucus lipids (2,3) have not distinguished between these sources. Gibbons' method of fractionating mucus (8) was modified to permit the centrifugation of the cellular debris. Subsequently it was found that a second fine particulate fraction, apparently containing the MGM, could also be separated. Samples of asthmatic mucus were dissolved in saturated $CaCl_2$/ethanol 9:1 v/v. The solutions were dialyzed in 0.25 M sucrose and centrifuged at 800 G for 10 min to remove cells and heavy debris (fraction 1). The supernatant was centrifuged at 20 kG for 30 min, which cleared nearly all of the opalescence and gave a grey pellet (fraction 2). The soluble glycoproteins in the supernatant were precipitated by bringing the solution to ca. 0.8 M ammonium sulfate. Centrifugation separated the precipitate (fraction 3) and the supernatant (fraction 4). Lipids were extracted from these fractions from three lots of mucus and tested for vitamin A by the methods described previously. The results are listed in Table II.

Under the light microscope, fraction 1 consisted of intact cells and nuclei but fraction 2 was amorphous. Electron microscopy showed that fraction 2 contained a large number of vesicles, 0.1-0.25 μ in diameter, and fine debris. Fractions 1 and 2 contained 95% or more of the total lipid, of which fraction 2 contributed 33-49%. Fractions 3 and 4 contained only traces of lipid, presumably from remnants of particulate matter not cleared by centrifuga-

tion. The yield of carotenoids, found principally in fraction 1, was markedly less than had been obtained by extraction of whole mucus from this patient, indicating that considerable autoxidation had taken place. To allow for these losses, fractions 1 and 2 were pooled from three lots of fractionated mucus before being analyzed for vitamin A. Retinyl esters were detected in fraction 2 by TLC mobility, fluorescence, and TFA color response, but the amount was too small to be quantitated. No vitamin A was found in fraction 1.

Aside from the exfoliated epithelial cells, the MGM appears to be the only other likely source of lipids in mucus. Neutra and Leblond (7) have clearly demonstrated the presence of these membranes in the lumen after secretion. Mucus will therefore contain large numbers of MGM which, being a relatively large structural lipoprotein, would sediment at moderate speeds. The distribution of lipids in mucus supports this view, for fractions 1 and 2 contained nearly all of the mucus lipid. Together they yielded the normal amount of lipid previously found in whole mucus from this patient, indicating complete extraction. The small vesicles mixed with fine debris seen in fraction 2 appear to be the MGM which have vesicularized during treatment with $CaCl_2$ and subsequent dialysis. This fraction is currently under further study.

An interpretation of these findings must consider the possibility that the presence of vitamin A in mucus is accidental and not functional. It could be argued that it would be found in virtually any tissue, but it should be noted that vitamin A was not found in the cellular fraction, even though the lipid content of this fraction was somewhat greater than that of the MGM fraction. The possibility that serous exudation contributed the vitamin A is discounted by the fact that it was found principally as retinyl esters rather than retinol, the form in which it occurs in the blood. Finally, the well known requirement of vitamin A for mucus secretion and its presence in mucus together comprise a striking coincidence which suggests a functional role.

These results raise the possibility that vitamin A is not converted into some active metabolite but is carried, essentially unchanged, in the secretion in which it played an important role. The only change is in its esterification. Similar esterification of retinol has been widely noted and extensively studied in the absorption of vitamin A from the intestine (9). The lack of any gross changes in vitamin A and its presence in the fraction containing the MGM are consistent with the role in membrane fusion proposed by Lucy (10). Membrane fusion is a critical step in secretion, and it may be the point at which vitamin A becomes essential to mucus secretion.

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ACKNOWLEDGMENTS

Mucus samples were contributed by E.M. Czochanska and P. Jacques.

REFERENCES

1. Wolbach, S.B., and P.R. Howe, J. Exp. Med. 42:753 (1925).
2. Lewis, R.W., Lipids 5:947 (1970).
3. Lewis, R.W., Ibid. 6:859 (1971).
4. Neeld, J.B., and W.H. Pearson, J. Nutr. 79:454 (1963).
5. Hoch, H., and R. Hoch, Brit. J. Exp. Pathol. 27:316 (1946).
6. Yeung, D.L., and M.J. Veen-Baigent, Can. J. Physiol Pharmacol. 50:48 (1972).
7. Neutra, M., and C.P. Leblond, J. Cell Biol. 30:119 (1966).
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9. Mahadevan, S., P.S. Sastry and J. Ganguly, Ibid. 88:534 (1963).
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[Received November 20, 1972]

Hydrolysis of Linoleate Geometric Isomers by *Geotrichum candidum* Lipase[1]

ABSTRACT

Lipase (EC 3.1.1.3) from the microorganism *Geotrichum candidum* preferentially hydrolyzes *cis*-9 18:1 and *cis,cis*-9,12 18:2 from triacylglycerols, largely ignoring all other positional isomers of *cis* 18:1 as well as *trans*-9 18:1. To obtain additional information about the specificity of the enzyme, two triacyl-

[1]Scientific contribution No. 535, Agricultural Experiment Station, University of Connecticut.

tion. The yield of carotenoids, found principally in fraction 1, was markedly less than had been obtained by extraction of whole mucus from this patient, indicating that considerable autoxidation had taken place. To allow for these losses, fractions 1 and 2 were pooled from three lots of fractionated mucus before being analyzed for vitamin A. Retinyl esters were detected in fraction 2 by TLC mobility, fluorescence, and TFA color response, but the amount was too small to be quantitated. No vitamin A was found in fraction 1.

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An interpretation of these findings must consider the possibility that the presence of vitamin A in mucus is accidental and not functional. It could be argued that it would be found in virtually any tissue, but it should be noted that vitamin A was not found in the cellular fraction, even though the lipid content of this fraction was somewhat greater than that of the MGM fraction. The possibility that serous exudation contributed the vitamin A is discounted by the fact that it was found principally as retinyl esters rather than retinol, the form in which it occurs in the blood. Finally, the well known requirement of vitamin A for mucus secretion and its presence in mucus together comprise a striking coincidence which suggests a functional role.

These results raise the possibility that vitamin A is not converted into some active metabolite but is carried, essentially unchanged, in the secretion in which it played an important role. The only change is in its esterification. Similar esterification of retinol has been widely noted and extensively studied in the absorption of vitamin A from the intestine (9). The lack of any gross changes in vitamin A and its presence in the fraction containing the MGM are consistent with the role in membrane fusion proposed by Lucy (10). Membrane fusion is a critical step in secretion, and it may be the point at which vitamin A becomes essential to mucus secretion.

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[1]Scientific contribution No. 535, Agricultural Experiment Station, University of Connecticut.

TABLE I

Fatty Acid Composition of Products from Hydrolysis of Two Triacylglycerols Containing Geometric Isomers of Linoleate by *Geotrichum candidum* Lipase

	9,12 18:2 Isomer, wt%			
	Triacylglycerol 1		Triacylglycerol 2	
Product	*cis,cis*	*trans,trans*	*cis,trans*	*trans,cis*
Original triacylglycerol	48	52	46	54
Residual triacylglycerol	35	65	48	52
Free fatty acids	84	16	45	55
Diacylglycerols	29	71	50	50
Monoacylglycerols	Trace[a]	90+	44	56

[a]Peaks were small, but only a trace of *cis,cis* isomer was observed.

glycerols were prepared and utilized as substrates. The lipase hydrolyzed 85% *cis,cis*-9,12 18:2 and 15% *trans,trans*-9,12 18:2 from the triacylglycerol, containing ca. 50% of each acid. From the triacylglycerol containing 46.3% *cis,trans*-9,12 18:2 and 53.7% *trans,cis*-9,12 18:2, 44.8 and 55.2% of the two acids were hydrolyzed. Therefore the enzyme discriminated against the *trans,trans* isomer but not between the *cis,trans* and *trans,cis* isomers.

INTRODUCTION

Lipase (EC 3.1.1.3, glycerol-ester hydrolase) from the microorganism *Geotrichum candidum* is highly specific for fatty acids with *cis*-9 and *cis,cis*-9,12 unsaturation releasing only small quantities of saturated acids, positional isomers of 18:1 other than Δ9 and of elaidic acid (1,2). On the basis of these findings we surmised that the enzyme should differentiate between *cis,trans*- and *trans,cis*-9,12 18:2s, hydrolyzing the former and not the latter from a triacylglycerol. We also believed that linoelaidate, 9,12-*trans,trans* 18:2, would not be digested by the enzyme. To test our hypotheses, we prepared two triacylglycerols—one from the isomers *cis,trans* 18:2 and *trans,cis* 18:2, and one from the acids *cis,cis* 18:2 and *trans,trans* 18:2. These substrates were hydrolyzed with *G. candidum* lipase and the acids in the products of lipolysis identified.

MATERIALS AND METHODS

The procedures, with the exception of gas liquid chromatography, have been described (2). Two digestions were made of each substrate. The methods employed by synthesis would yield approximately random placement of the acids. The linoleate and linoelaidate were obtained from The Hormel Institute and the two *cis,trans* isomers from the Northern Regional Research Laboratory.

Methyl esters of the acids from the products of lipolysis and of an added internal standard of 17:0 were determined in a Barber-Colman chromatograph at 190 C. The instrument was equipped with a column, 150 ft x 0.01 in. ID, coated with polyphenyl ether. Although separation of the two *cis,trans* isomers was incomplete, it was possible to determine the amount of each.

The amounts of each 18:2 isomer in all products of lipolysis and the original triacylglycerols are listed in Table I. As expected, *trans,trans* 18:2 was little hydrolyzed compared to *cis,cis* 18:2. The free fatty acids contained 84.5% of the *cis,cis* isomers, whereas the *trans,trans* isomer accumulated in the rest of the products; this is clear evidence of differentiation between the two isomers.

The results from the hydrolysis of the mixed *cis,trans* isomers, also presented in Table I, were surprising because there was little or no differentiation. Since the quantities of the two isomers were about the same in all products of lipolysis as in the original substrate, no discrimination was indicated. We have no explanation for this astonishing phenomenon, but after a comparison of molecular models we offer the following hypothesis.

In the 18:2 isomers that were digested readily the hydrogens on the 9, 10, 12 and 13 carbons can have the following conformations: *cis,cis,* all on one side, and *cis,trans* and *trans,cis,* three on one side, one on the other. Since the *trans,trans* isomer, which can only have two hydrogens on one side and two on the other, was poorly hydrolyzed, we have hypothesized that 18:2 isomers are digested by *G. candidum* lipase most readily if there are at least three hydrogens on one side of the molecule and the molecule contains one or more *cis* kinks.

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REFERENCES

1. Jensen, R.G., Prog. Chem. Fats Other Lipids 11:347 (1971).
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[Revised manuscript received March 21, 1973]

Occurrence of (+)-Erythro-2,3-Dihydroxyhexacosanoic Acid in Cerebrin from the Yeast *Saccharomyces cerevisiae*[1]

ABSTRACT

(+)-Erythro-2,3-dihydroxyhexacosanoic acid was found in a mixture of cerebrins (ceramides containing sphingosines and phytosphingosines) from the yeast *Saccharomyces cerevisiae*. The structure was deduced on the basis of elemental analyses, gas liquid chromatography and thin layer chromatography of the appropriate derivatives of the acid and its oxidation products.

The long chain 2,3-dihydroxy fatty acids have not attracted much attention from previous researchers. To the best of our knowledge 2,3-dihydroxytetracosanoic acid is the only member of this series found in nature. It was reported in 1952 by Oda (1) as a constituent of hydroxycerebrin which was isolated from the mycelium of surface-cultivated penicillin-producing mold Q 176. Cerebrins are mixtures of ceramides containing the long chain bases sphingosines and phytosphingosines, and the amide-linked long chain fatty acids, predominantly of the hydroxy series. This communication is a report on the occurrence of (+)-erythro-2,3-dihydroxyhexaconsanoic acid in cerebrin from *Saccharomyces cerevisiae*.

Crude yeast cerebrin was supplied by N.V. Philips-Roxane, Pharmaceutisch-Chemische Industrie DUPHAR, Amsterdam. Methanolysis of 10 g cerebrin (which contained considerable quantities of ergosterol and other impurities) according to Reindel and coworkers (2) and subsequent alkaline hydrolysis gave a mixture of long chain fatty acids (2.95 g). The total hydroxy fatty acids (HFA, 1.65 g) were separated from the nonhydroxy ones via copper chelates (1.78 g) by using a method described previously (3,4). The methyl esters were prepared by adding excess diazomethane in diethyl ether to methanol solution of the hydroxy acids. Thin layer chromatography (TLC) on Silica Gel G (E. Merck, A.G., Darmstadt), 250 μ thick (solvent system chloroform-methanol 19:1.5 v/v, detection with ammonium molybdate-perchloric acid spray reagent [5]), showed two main spots corresponding to the fast moving (Rf 0.90) monohydroxy fatty acid fraction (MHFA) and the slow moving (Rf 0.66) dihydroxy fatty acid fraction (DHFA).

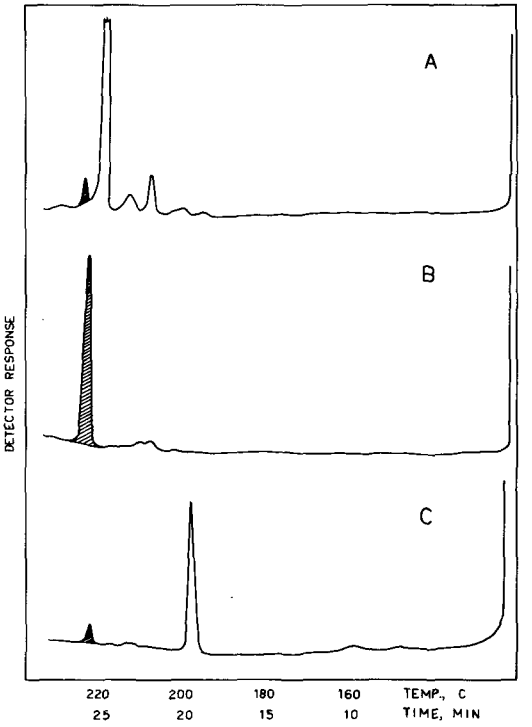

FIG. 1. Gas chromatographic analysis of fatty acids of yeast cerebrin and their degradation products (in form of acetylated methyl esters). (A) Total HFA; (B) (+)-erythro-2,3-dihydroxyhexacosanoic acid (DHFA) hatched area; (C) products of periodate-permanganate oxidation of DHFA (tetracosanoic acid).

[1]Studies in the sphingolipids series, XXXIV. Presented in part at the Meeting of Croatian Chemists, Zagreb, February 1971.

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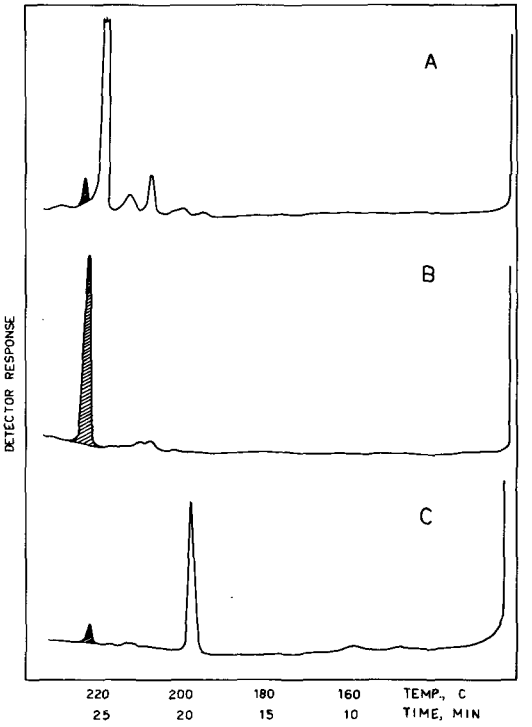

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[1]Studies in the sphingolipids series, XXXIV. Presented in part at the Meeting of Croatian Chemists, Zagreb, February 1971.

The mixture of HFA methyl esters (1.68 g) was dissolved in chloroform and chromatographed on a column of Florisil (30 g, 60/100 mesh, Floridin Co., Tallahassee, Fla., as purchased). The chloroform eluates contained MHFA methyl esters (predominantly methyl 2-hydroxyhexacosanoate), while chloroform-methanol 95:5 eluted DHFA methyl esters. TLC was used to monitor the column separation. Fractions containing chromatographically pure DHFA esters (Rf 0.66) were collected. Glistening leaflets, 0.12 g, 7.1% of the total HFA esters, mp 95-96 C; found: C 73.05, H 11.99. $C_{27}H_{54}O_4$ requires: C 73.25, H 12.29%. Alkaline hydrolysis afforded the free acid, mp 104 C, $[\alpha]_D^{22}$ = + 2.45 C (in pyridine); found: C 72.63, H 11.95. $C_{26}H_{52}O_4$ requires: C 72.84, H 12.23%. The chemical structure and the stereochemical assignment of the pure DHFA were investigated by means of gas liquid chromatography (GLC), TLC (6) and oxidative degradations.

Both total HFA and pure DHFA methyl esters were acetylated by refluxing with acetyl-chloride for 1 hr and evaporating the solution to dryness. The acetylated methyl esters were analyzed by GLC. A Perkin-Elmer Model F 20 instrument equipped with a hydrogen flame detector, containing a glass column (2 m x 2.7 mm) packed with 1% SE-30 on Chromosorb G 80/100 mesh, was used. This was temperature-programed from 120 to 250 C at 2 C/min during analyses, and nitrogen was used as carrier gas; flow rate was 50 ml/min. The acids were identified by comparing their programed temperatures to those of authentic standards and by plotting the temperatures against carbon numbers. GLC of total HFA and DHFA (in the form of acetylated methyl esters) is shown in Figure 1A and B. The hatched area represents (+)-erythro-2,3-dihydroxyhexacosanoic acid. The same retention time was obtained with the synthetic acid prepared in this laboratory by hydroxylation of *trans*-2-hexacosenoic acid with both alkaline permanganate (threo-isomer) and peracetic acid (erythro-isomer). Tetracosanoic acid and 2-hydroxyhexacosanoic acid were also preparations from this laboratory.

The periodate-permanganate oxidation (7) of DHFA methyl ester yielded a mixture of fatty acids which was esterified and acetylated as described above. The main product was tetracosanoic acid in admixture with some unreacted DHFA as shown by GLC (Fig. 1C).

The erythro-configuration was assigned to (+)-2,3-dihydroxyhexacosanoic acid by means of TLC on Silica Gel G impregnated with boric acid; the solvent system was chloroform-methanol 19:1 (6). Boric acid forms complexes more readily with threo-glycols than with erythroglycols. The more polar erythro-isomer migrated more slowly (Rf 0.62) than the less polar boric acid complex of the threo-isomer (Rf 0.69).

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ACKNOWLEDGMENT

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REFERENCES

1. Oda, T., J. Pharmacol. Soc. Japan 72:136 (1952).
2. Reindel, F., Liebigs Ann. 480:76 (1930); F. Reindel, A. Weickmann, S. Pickard, K. Luber and P. Turula, Ibid. 544:116 (1940).
3. Radin, N.S., J.R. Brown and F.B. Lavin, J. Biol. Chem. 219:977 (1956).
4. Palameta, B., and M. Prošteník, Tetrahedron 19:1463 (1963).
5. Wagner, H., L. Hörhammer and P. Wolf, Biochem. Z. 334:175 (1961).
6. Kljaić, K., and M. Prošteník, Bull. Sci., Cons. Acad. Sci. Arts RSF Yougoslavie, Sect. A, 18:5 (1973).
7. Lemieux, R.U., and E. von Rudloff, Can. J. Chem. 33:1701 (1955); T.C. Lo Chang and C.C. Sweeley, J. Lipid Res. 3:170 (1962).

[Revised manuscript
received March 10, 1973]

ERRATUM

Due to a printer's error, the references to "Effect of Soy Sterols on Cholesterol Synthesis in the Rat" (Lipids 8:42[1973]) were poorly reproduced. They are reprinted below.

REFERENCES

1. Peterson, D.W., Proc. Soc. Exp. Biol. Med. 78:143 (1951).
2. Sylvén, C., and B. Borgström, J. Lipid Res. 10:179 (1969).
3. Klauda, H.C., and F.M. Quackenbush, Lipids 5:142 (1970).
4. Konlande, J.E., and H. Fisher, J. Nutr. 98:435 (1969).
5. Katz, M., I. Bartov, P. Budowski and A. Bondi, J. Nutr. 98:1141 (1970).
6. Dietschy, J.M., and M.D. Siperstein, J. Lipid Res. 8:97 (1967).
7. Cayen, M.N., J. Lipid Res. 12:482 (1971).
8. Grundy, S.M., E.H. Ahrens, Jr. and J. Davignon, Ibid. 10:304 (1969).
9. Dietschy, J.M., J. Clin. Invest. 47:286 (1968).

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SYMPOSIUM: BIOLOGICAL SIGNIFICANCE OF AUTOXIDIZED AND POLYMERIZED OILS

Presented at the JOCS-AOCS Joint Meeting, Los Angeles

I. HARA, Chairman

H. KAUNITZ, Cochairman

Exacerbation of Heart and Liver Lesions in Rats by Feeding of Various Mildly Oxidized Fats[1]

HANS KAUNITZ and **RUTH E. JOHNSON**, Department of Pathology, Columbia University, New York, New York 10032

ABSTRACT

Groups of 40 male Charles River rats were fed diets containing cottonseed oil, olive oil, corn oil, soybean oil, coconut oil, chicken fat, beef fat, butter oil, lard and saturated medium chain triglycerides. The fats were fed fresh and after 40 hr aeration at 60 C, which hardly changed peroxide values. In addition, fresh and aerated soybean oil and lard were fed to W/Fu rats. Body weights and life span were significantly influenced by the kind of fat fed, but not by aeration. Many hearts exhibited unspecific focal myocarditis and focal fibrosis. The latter was graded in a blind test, which revealed highly significant differences in the incidence of severe lesions; those fed corn oil had the most, followed by cottonseed oil, soybean oil, olive oil, beef fat, saturated medium chain triglycerides, butter, chicken fat and lard, in that order. Feeding of aerated fat resulted in an increased incidence with six of the eight fats. The W/Fu rats had lower incidences, but those fed soybean oil had more than those fed lard, and aeration led to a higher incidence. Some heart sections stained with Light Green SF Yellowish revealed areas of muscle fibrils that did not accept the stain, probably as a conse-

[1]One of seven papers presented in the symposium "Biological Significance of Autoxidized and Polymerized Oils," JOCS-AOCS Joint Meeting, Los Angeles, April 1972.

INDEX

TABLE I

Analyses of One Batch of Fats Fed to Rats from Weaning

Oil	Peroxide value,[a] Meq/kg	Epoxide value,[b] Meq oxirane oxygen/kg	Carbonyl value,[c] Meq/kg		
			Unsaturated	Saturated	Total
MCT[d]	0.1	0	0.4	1.7	2.1
Olive oil					
Fresh	15.2	4	3.2	6.1	9.3
Aerated	18.5	6	3.2	7.4	10.1
Corn oil					
Fresh	1.9	3	7.3	4.1	11.4
Aerated	4.0	3	7.1	3.6	10.7
Cottonseed oil					
Fresh	7.8	5	6.0	0.8	6.8
Aerated	6.7	9	7.1	3.4	10.5
Soybean oil					
Fresh	1.0	0	3.0	2.6	5.6
Aerated	2.3	0	2.9	3.0	5.9
Irradiated	2.3	0	2.1	7.2	9.3
Beef fat					
Fresh	4.9	7	2.8	8.1	10.9
Aerated	3.5	3	2.2	4.9	7.1
Butter fat					
Fresh	1.0	0	1.7	8.4	10.9
Aerated	2.5	0	1.7	6.7	8.4
Chicken fat					
Fresh	1.9	1	0.6	2.9	3.5
Aerated	4.3	1	0.6	3.5	4.1
Lard					
Fresh	2.0	1	1.7	4.7	6.4
Aerated	2.3	10	1.9	5.0	6.9
Irradiated	1.7	0	1.3	8.8	10.1

[a]AOCS Official Method Cd 8-53.

[b]Fioriti et al., JAOCS 43:487 (1966).

[c]Fioriti, JAOCS 42:743 (1965).

[d]Saturated medium chain triglycerides.

quence of cellular damage. Higher incidences of this lesion were associated with the same fats as was severe fibrosis, and feeding of aerated fats led to higher incidences. Many livers revealed marked proliferation of bile ducts. The groups fed cottonseed, soybean, olive and corn oils had higher incidences of severe lesions, and feeding of the oxidized oils led to still higher incidences. None of the results appeared to be associated with the fatty acid composition of the fats, which suggested that these long term effects may have been due to minor constituents in the individual fats.

For the last 25 years, various studies have been undertaken to determine the extent to which changes induced in fats by thermal or oxidative treatment would produce toxic effects in animals fed these fats. In the early 1950's, feeding experiments were carried out with fats that had undergone rather severe damage by heat or oxidation (1,2). Interest centered on separation of broad classes of compounds produced in such fats. Some of these groups of compounds were so toxic that feeding studies were necessarily of short duration. Rats fed these materials exhibited diarrhea, edema of the gut, and enlarged livers and kidneys (3). All of this accumulating knowledge had social consequences: processors of such foods as potato chips, French fried potatoes and other deep-fried foods became much more careful with regard to how long they used any one batch of oil, and, at the same time, manufacturers of various fats and oils and of foods containing these oils routinely included antioxidant in their products.

Further investigations dealt with possible effects produced by fats after ordinary cooking procedures or after storage. In general, it was found that growth and life span of animals fed such fats were not adversely affected (4-8). However it was reported that rats fed mildly oxidized beef fat appeared to have less linoleate in their depots than those fed fresh beef fat (9). Furthermore it was observed that feeding of

TABLE II

Composition of Purified Diet

A.

Component	%
Vitamin-free test casein[a]	30
Dextrose[b]	44
Salt mixture (Bernhart & Tomarelli)[a]	4
Cellulose (Alphacel)[a]	2
Fat	20

B.

Component	mg/kg	Component	mg/kg
Choline dihydrogen citrate[a]	1000	Vitamin D_2[a]	0.5
Inositol[a]	1000	Thiamin-HCl	4
p-Aminobenzoic acid[a]	300	Pyridoxine-HCl	8
Nicotinamide[a]	100	Riboflavin	8
Vitamin K (Synkayvite)	10	Ca pantothenate	20
α-Tocopherol acetate	100	Folic acid	5
Free α-tocopherol	10	Biotin	0.05
β-Carotene	5	Ascorbic acid	50
		Vitamin B_{12} (0.1% trituration in mannose)	10

[a]Nutritional Biochemicals Corp., Cleveland, Ohio. All other vitamins contributed by Hoffmann-La Roche Inc., Nutley, N.J.

[b]Corn Products Co., Englewood Cliffs, N.J.

mildly oxidized fats led to increased vitamin requirements (10), as had been shown previously for more highly oxidized fats (11).

In an earlier long term feeding study carried out in our laboratory, with eight fresh and mildly oxidized vegetable and animal fats fed to rats in a low iodine diet, the average life spans of the groups fed fresh vegetable oils were shorter than those of the groups fed fresh animal fats, and feeding of the oxidized forms of three of the oils (olive, corn and soybean) resulted in longer life spans (7). Furthermore there was some suggestion that the various fats exerted other effects which were unrelated to their fatty acid composition, and the hypothesis was put forth that fats, particularly vegetable oils, even after refining, may contain biologically active substances in their nontriglyceride fractions which exert long term effects.

Because our early experiment was complicated by a low iodine diet and periodic sacrifices for lipid chemistry, a more comprehensive feeding study was undertaken with larger groups of rats, all of which could be observed until they died of natural causes.

EXPERIMENTAL PROCEDURES

Fats and Oils

The fats studied were cottonseed oil, olive oil, corn oil, soybean oil, coconut oil, chicken fat, beef fat, butter oil, lard and a randomized mixture of saturated medium chain triglycerides (MCT), (mainly C_8 and C_{10}). All of these fats had been prepared for human consumption, and three different batches of each were used in the course of the experiment. Half of each batch (one-third in the case of soybean oil and lard) was aerated at a rate of 1 liter of air per gallon per minute for 40 hr at 60 C. In addition, part of the lard and soybean oil was canned according to FDA specifications for irradiation of foods and was given 4.5 Mrad of gamma irradiation. These treatments brought about only very slight changes in peroxide, epoxide and carbonyl values (Table I). The fats were incorporated in a highly purified diet, the composition of which is given in Table II.

Animals

The main study was carried out on Charles River (CD) male rats. All fresh, aerated and irradiated fats were fed to groups of 40 rats each from the age of 28 days. In addition, four groups of 40 male rats of the smaller W/Fu strain were fed fresh and aerated lard and soybean oil. The rats were assigned to the individual groups on the basis of stratified body weights; all groups had the same weight range and the same average weights.

The rats were housed, two per cage, on racks holding 20 cages (10 with rats fed a fresh fat and 10 with rats fed the corresponding aerated

TABLE III

Relation of Average Body Weight at Early Age to Eventual Average Life Span in Groups of Charles River Rats Fed Various Fats

Oil	Fresh		Aerated	
	Average wt at 238 days, g	Average life span, days	Average wt at 238 days, g	Average life span, days
Coconut oil	738±15.8[a]	592±28.4[a]	735±13.2	549±29.5
Chicken fat	723±17.2	613±19.7	712±18.1	655±23.5
Butter	703±19.2	626±21.2	694±12.5	640±18.7
Cottonseed oil	701±12.9	641±24.2	725±23.2	650±25.0
Corn oil	691±15.5	652±21.2	687±14.0	664±22.2
Lard	688±16.8	639±20.3	697±26.6	665±20.7
Soybean oil	678±12.5	670±22.1	683±12.2	635±21.9
Olive oil	646±10.7	665±17.9	662±12.5	642±29.3
Beef fat	626±14.1	703±19.2	650±16.0	690±17.1
MCT[b]	614±10.7	703±24.5		
	P < .001	P = .005	P = .01	P < .01

[a]Standard error.

[b]Saturated medium chain triglycerides.

fat). The other halves of the same groups were housed on a second rack on the opposite side of the room, and the racks were rotated frequently. All rats of one strain were kept in the same room.

The groups fed coconut oil were placed in the experiment one year after the rest. The coconut oil diets initially contained a supplement of 0.5% of linoleic acid, but early mortality among these animals suggested that this was too low. When the rats were 9 months of age, the linoleic acid supplement was raised to 1.5% and the high death rate subsided.

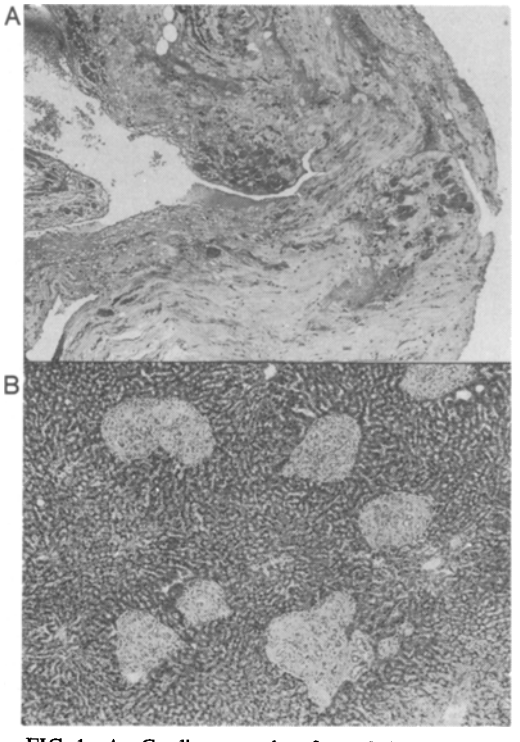

FIG. 1. A: Cardiac muscle of rat fed cottonseed oil. Muscle fibers are largely replaced by fibrous tissue (H & E; x 90). B: Proliferation of bile ducts in liver of rat fed corn oil (H & E; x 70).

The animals were inspected daily and any dead or moribund were autopsied as soon as possible. At least 10 tissues were fixed in Bouin's solution for histology, together with any tissues suspected of being abnormal. Sections of all tissues were stained with hematoxylin and eosin, and sections of hearts were also stained with Carr's chloranilic acid stain for calcium with Light Green SF Yellowish as counterstain.

The data for each animal were coded and key punched into an 80 column IBM card, which was used as input to an IBM 360/91 computer at the Columbia University Computer Center.

RESULTS AND DISCUSSION

When the rats had been on their diets for three months, there was a spread of 60 g in the median weights of the Charles River groups fed fresh fats, and this difference increased to ca. 200 g at the age of 605 days. In addition to intergroup differences, there was a considerable spread in the body weights of an individual group (over 300 g at 133 days of age in the group fed fresh corn oil). At 238 days of age, there was a spread of over 100 g in the average weights of the groups fed fresh fats (Table III), and an analysis of variance showed that the intergroup differences were significant.

Because of the frequently cited inverse relationship between early overweight and even-

TABLE IV

Observed and Expected Incidences of Severe (3+ and 4+) Cardiac Fibrosis and of Marked Discoloration after Heart Sections Were Stained with Light Green SF Yellowish

Oil	Fibrosis		Resistance to green stain	
	Fresh	Oxidized	Fresh	Oxidized
Corn oil	26/14.5=1.80	25/16.0=1.57	17/7.9=2.16	22/8.9=2.47
Cottonseed oil	20/16.3=1.23	24/15.7=1.53	12/8.0=1.50	14/8.2=1.71
Soybean oil	16/15.6=1.03	22/15.1=1.46	9/8.3=1.09	15/8.3=1.80
	(Irrad.)	18/15.3=1.17[a]	(Irrad.)	4/8.3= .48[a]
Olive oil	16/15.9=1.01	18/15.7=1.15	13/9.1=1.42	16/7.9=2.02
Beef fat	16/16.9= .94	20/17.0=1.17	6/9.6= .62	10/9.9=1.01
MCT[b]	16/17.6= .91[a]		2/8.1= .25[a]	
Butter	11/15.3= .72	10/15.3= .65	3/6.9= .43	5/8.5= .59
Chicken fat	10/14.5= .69	13/14.5= .84	5/7.2= .69	8/9.1= .88
Lard	9/14.7= .61	19/16.5=1.15	3/8.4= .36	4/9.6= .41
	(Irrad.)	12/16.3= .74[a]	(Irrad.)	2/8.4= .24[a]
Chi square =	23.89	26.48	27.89	53.40
P (7 df)	$\ll .01$	$< .001$	$\ll .001$	$\ll .001$

[a]Not in chi square calculations.

[b]Saturated medium chain triglycerides.

tual life span, we selected the body weights at 238 days as indicative of early overweight. Table III shows highly significant differences among the average life spans of the groups fed fresh fats and an inverse relationship between weight and life span. The comparatively short average life span of the group fed coconut oil is due partly to the early deaths in the initial period of linoleate deficiency. The groups fed beef fat and MCT consistently had lower body weights, and only 6 fed beef fat and 10 fed MCT had died by the age of 600 days.

Feeding of oxidized fats did not result in any significant alteration in body weight or life span. There were highly significant intergroup differences with regard to weight and life span, but no apparent inverse relationship between the two.

The W/Fu rats had longer average life spans, ranging from 695 to 745 days for the four groups. The differences were not significant.

Degenerative Lesions

The heart was the most frequent site of degenerative changes. The latter included focal, unspecific myocarditis, fibrosis and, occasionally, calcium deposits. The myocarditis was characterized by invasion of focal areas of the heart by modified histiocytes and destruction of muscle fibrils. Fibrous tissue replaced some of the muscle fibers, as shown in Figure 1A. The severity of these fibrotic changes was evaluated in a blind test on a scale of 0 to 4+ for statistical treatment. It was apparent that the incidence of severe focal fibrosis was age-dependent, increasing from 26% of those dying between 400 and 500 days to 80% of those dying over 900 days of age; therefore any comparison of groups would have to take into account their different mortality patterns. We adapted the age-specific analysis used by Ross and Bras (12), in comparing tumor incidences in different dietary groups with different average life spans. We pooled the data from all groups fed fresh fats and established incidence factors for successive 100 day periods (number of cases of severe fibrosis per number of rats dying during that period); these age-specific incidence factors were then applied to an individual dietary group, to arrive at an estimate of the number of severe cases to be expected in each 100 day period if the group conformed to the average for the whole population. These "expected" incidences were added, to arrive at the expected incidence for the whole experimental period, and this could be compared with the incidence actually observed. The ratio of observed to expected incidence was a value that lent itself to intergroup comparisons.

The observed and expected incidences of severe (3+ and 4+) cardiac fibrosis are shown in Table IV. Among the groups fed fresh fats, the observed incidence ranged from 180 to 61% of that expected on the basis of the population as a whole, and the intergroup differences were highly significant (Chi square = 23.9; with 7 df, $P < .01$). The groups fed coconut oil have been omitted because of the higher early incidence of cardiac damage associated with the initial transitory linoleate deficiency. The groups fed vegetable oils, particularly corn and cottonseed oil, had more focal fibrosis than did those fed animal fats, particularly chicken fat and lard.

In order to bring out any possible effect of oxidation and strain, the age-specific incidence

TABLE V

Observed and Expected Incidences of Severe (3+ and 4+) Bile Duct Proliferation

Oil	Fresh	Aerated	Irradiated
Cottonseed oil	15/ 9.2=1.62	21/ 9.0=2.34	
Soybean oil	14/ 9.7=1.44	16/ 8.0=2.00	15/8.3=1.81
Olive oil	11/ 9.1=1.21	9/ 8.3=1.08	
Corn oil	12/10.0=1.20	17/10.0=1.70	
Butter	10/ 8.5=1.17	3/ 9.5= .32	
Beef fat	9/10.2= .89	10/ 9.9=1.01	
Chicken fat	7/ 8.0= .88	4/ 8.2= .49	
MCT[a]	8/ 9.9= .81		
Lard	5/ 9.6= .52	5/ 9.1= .55	3/8.3= .36
Coconut oil	2/ 8.9= .22	3/ 7.7= .39	
Chi square = (8 df)	18.22	54.555	
	P = .02	P ≪ .001	

[a]Saturated medium chain triglycerides, omitted from chi square calculation.

factors derived from the whole population of CR rats fed fresh fats (except coconut oil) were used to calculate the expected incidences in the groups fed oxidized and irradiated fats and in the groups of W/Fu rats. The table shows that with six of the eight fats, intake of the oxidized fat was associated with an increase in the incidence of severe fibrosis, whereas irradiated fats were associated with only a slight increase.

The observed incidence of severe fibrosis among the W/Fu rats was much lower, although they lived somewhat longer and thus had higher expected incidences. Nevertheless, in this strain of rats, feeding of soybean oil was associated with a higher incidence than was lard, and oxidation led to an increase in the incidence in both groups.

Many of the heart sections stained with hematoxylin and eosin had deep blue areas suggestive of calcification. Therefore an additional section from each heart was stained with chloranilic acid (13) and counterstained with Light Green SF Yellowish stain. We were unable to find any of the brownish microcrystals formed between chloranilic acid and calcium, probably because the tissues had been fixed in Bouin's fluid, an acid fixative. However we did observe that the centers of many muscle fibrils were not green, but appeared light brown. Duplicate sections stained only with Light Green SF Yellowish revealed that these areas were evidently resistant to this stain (appearing only light yellow). Lesions of the myocardial fibrils, possibly similar to the one noted here, have been described by others. Wartman and Hill (14) described a change in older myocardial infarcts, in which dead muscle is not replaced by scar tissue and there is no obvious loss of architecture.

Since the discoloration noted by us seemed to vary in amount with age and with dietary fat, we graded the extent of this material on a scale of 0 to 4+ in a blind test; the data were given the same kind of age-specific analysis used in evaluating fibrosis, because the incidence of extensive involvement varied from 4% among rats dying between 400 and 500 days to 32% among those dying after 800 days.

Table IV shows the observed and expected incidences of marked discoloration. The observed incidences among the rats fed fresh fats varied from 216 to 36% of that expected on the basis of the population as a whole; the differences between groups were highly significant. It is apparent from the table that cardiac fibrosis and "muscle fibril damage" vary in nearly the same order from group to group.

The age-specific incidence factors derived from the population fed fresh fats were used to calculate the expected incidences in the groups fed oxidized and irradiated fats and in the W/Fu groups. The table shows that, in all groups, feeding of the oxidized fat increased the incidence over that seen in the group fed the corresponding fresh fat; irradiation, on the other hand, did not appear to have this effect (if anything, there was less involvement). In agreement with our observations on fibrosis, the W/Fu rats showed less fibril involvement. Among the W/Fu groups, those fed fresh soybean oil also had a higher incidence; and feeding of the oxidized lard, at least, led to an increase in incidence over that in the group fed fresh lard.

Renal lesions were present, to some extent, in most of our older rats. They were characterized by atrophy or dilatation of tubules and hyaline casts ("thyroidization") and by tubular calcification. Similar changes have been described by others (15,16). The glomerular

changes observed by Bras and Ross (17) were mild in our rats. Severe renal changes were nearly always associated with hypertrophy of the parathyroids. The severity of the renal lesions was graded on a scale of 0 to 4+ in a blind test, and the results were given an age-specific analysis. There were no statistically significant intergroup differences; if anything, there were somewhat fewer cases of severe kidney damage among those fed vegetable oils. Furthermore feeding of oxidized fats did not increase the incidence of this disease. There was no correspondence between the distribution of severe cardiac damage and severe kidney thyroidization among the various dietary groups.

Many livers of the Charles River rats had a characteristic lesion of the bile ducts. This is shown in Figure 1B. There were proliferation and distension of the bile ducts and accumulation of considerable amounts of connective tissue usually without inflammatory cells. We graded the liver sections as to the extent of this bile duct proliferation and analyzed the results by means of an age-specific analysis because this disease also increased with age. The observed and expected incidences of the severe form of this lesion are shown in Table V. There were significant differences among the groups, with the groups fed unsaturated vegetable oils having more lesions. Feeding of mildly oxidized vegetable oils induced a higher incidence, which was not the case with the animal fats. Irradiation appeared to have the same effect as oxidation. In view of the fact that there did not appear to be any biliary obstruction or inflammatory cells, it seems likely that the proliferation was caused by some circulating toxic material.

DISCUSSION

The various fats under study exerted long term nutritional effects which did not appear to be related to their fatty acid composition or triglyceride structure. Even such mild oxidation as was carried out in this experiment had effects on hearts and livers. It is true that most severe degenerative changes were brought on by the unsaturated fats, including olive oil, and that these changes were increased significantly by mild oxidation. However the effects of the oxidized fats occurred without an appreciable rise in the peroxide, carbonyl and epoxide values, which makes it seem unlikely that the effects were due to changes in the fatty acids. Therefore these results support our original hypothesis that pharmacologically active materials present in the nontriglyceride fraction may be responsible for some of the long term effects of fats and that these substances may be altered by mild oxidation, with reduction or production of toxic materials.

The fact that particularly the nontriglyceride fraction of vegetable oils may contain active materials can be assumed because of the practically limitless number of such materials in plants (18), many of which are fat soluble. Furthermore environmental contaminants were detected in some of our samples. One batch of chicken fat contained traces of chlorinated pesticides, while one batch of soybean oil contained 100 ppb polychlorinated biphenyls. Fats also may contain hormones given to animals in their feed; they frequently have added antioxidants, the biological effects of which are now under discussion. All of this may explain why different long term experiments often give different results and why future work purporting to study the effects of triglycerides should be carried out with carefully purified fats. Because of the uncertainty as to what contaminants may be present in any fat, there is at present little point in condemning one fat more than another. Improved processing of all fats for human consumption seems necessary.

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REFERENCES

1. Crampton, E.W., R.H. Common, F.A. Farmer, A.F. Wells and D. Crawford, J. Nutr. 49:333 (1953).
2. Kaunitz, H., C.A. Slanetz, R.E. Johnson, H.B. Knight, D.H. Saunders and D. Swern, Fed. Proc. 14:408 (1955).
3. Kaunitz, H., C.A. Slanetz, R.E. Johnson, H.B. Knight, D.H. Saunders and D. Swern, JAOCS 33:630 (1956).
4. Alfin-Slater, R.B., S. Auerbach and L. Aftergood, Ibid. 36:638 (1959).
5. Artman, N.R., and J.C. Alexander, Ibid. 45:643 (1968).
6. Poling, C.P., W.D. Warner, P.E. Mone and E.E. Rice, J. Nutr. 72:109 (1960).
7. Kaunitz, H., R.E. Johnson and L. Pegus, Proc. Soc. Exp. Biol. Med. 123:204 (1966).
8. Lang, K., Z. Ernaehrungswiss. 10:234 (1971).
9. Lea, C.H., and L.J. Parr, Brit. J. Nutr. 18:369 (1965).

10. Kummerow, F.A., C. Hemans and E.G. Perkins, Fed. Proc. 31:Abstr. 2674 (1972).
11. Kaunitz, H., R.E. Johnson and C.A. Slanetz, J. Nutr. 46:151 (1952).
12. Ross, M.H., and G. Bras, Ibid. 87:245 (1965).
13. Carr, L.B., O.N. Rambo and T.V. Feichtmeir, J. Histochem. Cytochem. 9:415 (1961).
14. Wartman, B., and W.T. Hill, in "Pathology of the Heart," Second edition, Edited by S.E. Gould, Charles C Thomas Publishers, Springfield, Ill., 1960, p. 497.
15. Wilens, S.L., and E.E. Sproul, Amer. J. Pathol. 14:201 (1938).
16. Saxton, J.A., and G.C. Kimball, Arch. Pathol. 32:951 (1941).
17. Bras, G., and M.H. Ross, Toxicol. Appl. Pharmacol. 6:247 (1964).
18. Leopold, A.C., and R. Ardrey, Science 176:512 (1972).

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Polymerization Inhibitors for Polyunsaturated Oils[1]

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ABSTRACT

Several edible anionic surfactants were found to function as polymerization inhibitors for safflower oil. The most effective additive was the sodium salt of phosphated mono- and diglycerides. Additional inhibitors included sodium diacetyltartaric acid esters of mono- and diglycerides, sodium stearoyl-2-lactylate, sodium stearyl fumarate, sodium succinoylated mono- and diglycerides, dioctyl sodium sulfosuccinate, and sodium sulfoacetate esters of mono- and diglycerides. It is suggested that these surfactants behave in a manner similar to methyl polysiloxane, as oxygen barriers at the oil-air interface. This view is supported by data showing that in the free acid, oil soluble form their functionality is minimal. But when they are neutralized to form the sodium salts their effectiveness is markedly enhanced.

INTRODUCTION

Methyl polysiloxane is frequently added to frying oils for the inhibition of oxidative polymerization. This material appears to form a protective film at the oil-air interface which limits access to oxygen (1). Although food and drug regulations permit its addition at levels of up to 10 ppm, it is so functional that an effect is obtained even below 1 ppm. Since it is an active defoamer, addition to shortenings designed primarily for baking must be avoided.

Although there appeared to be little incentive for development of silicone replacements, curiosity led us to test a series of edible anionic surfactants in heated safflower oil. Several of these were found to be quite functional in retarding the rate at which the oil polymerized. This paper represents an extension of earlier work in which certain sterols were shown to behave as polymerization inhibitors (2).

METHODS AND MATERIALS

The test method used here involved continuous heating of 100 g samples of safflower oil in 150 ml beakers on electric hot plates at 180±5 C for periods of up to 46 hr. The additives were dispersed in the oil prior to heating. In each test a control sample containing no additive was run concurrently.

The iodine value was chosen as a simple method for monitoring the rate of destruction of polyunsaturates. This technique was used by Waltking and Zmachinski (3), who demonstrated that the decrease in iodine value parallels the loss of polyunsaturates in heated vegetable oils. At the termination of the heating period, relative viscosity measurements were obtained by determining flow rates through a 50 ml pipet at room temperature.

Gas liquid chromatography of methyl esters was performed using a Perkin Elmer 900 gas chromatograph equipped with a dual flame

[1]One of seven papers presented in the symposium "Biological Significance of Autoxidized and Polymerized Oils," JOCS-AOCS Joint Meeting, Los Angeles, April 1972.

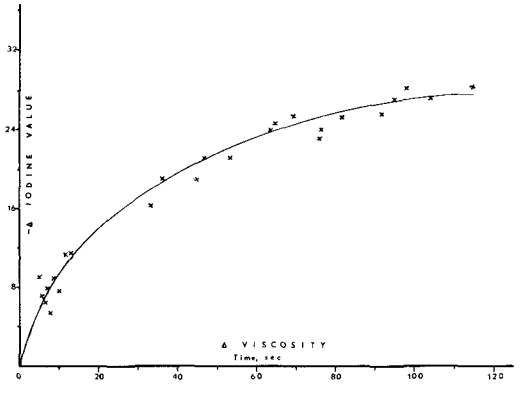

FIG. 1. Heated safflower oil samples.

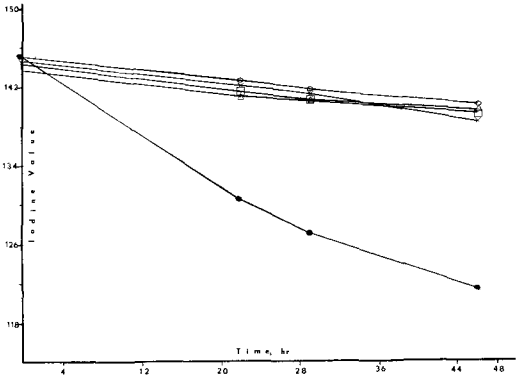

FIG. 2. Heating test on safflower oil. Phosphated mono- and diglycerides: • control; ○ 5ppm silicone oil; × 0.2%; □ 0.5%; and △ 1.0%.

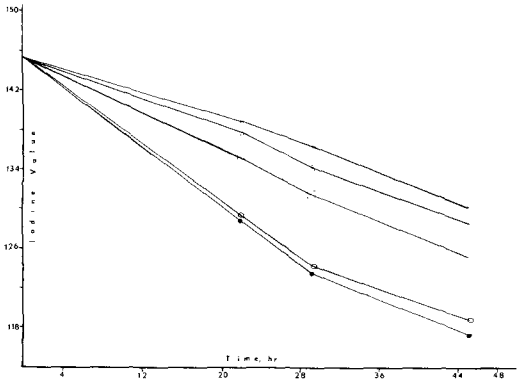

FIG. 3. pH of polymerization inhibitor for safflower oil. Diacetyl tartaric acid esters of mono- and diglycerides: • control; ○ pH 2.2; □ pH 4.2; △ pH 5.7; and × pH 7.2.

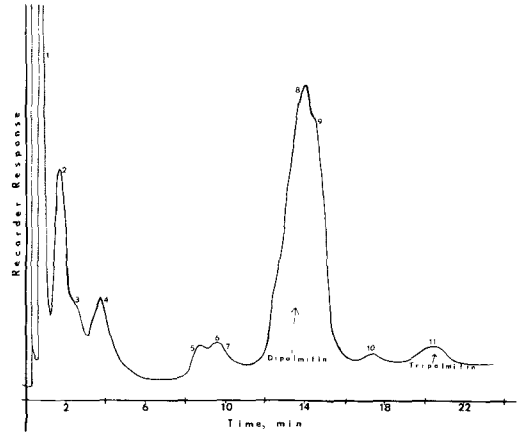

FIG. 4. Gas liquid chromatography of urea nonadducting fraction.

ionization detector. The column (10 ft x 1/8 in. OD) was 12% stabilized DEGS on 70/80 mesh Anakrom ABS.

Triglycerides and also the trimethylsilyl (TMS) derivatives of the urea nonadducting fatty acids were chromatographed using a Hewlett Packard 5750 gas chromatograph equipped with dual flame ionization detectors. This method has been described earlier (4).

Safflower oil was a product of the Pacific Vegetable Oil Co. The sodium salts of phosphated mono- and diglycerides, sodium sulfoacetate derivatives of mono- and diglycerides and diacetyltartaric acid esters of mono- and diglycerides were obtained from the Witco Chemical Corp. Sodium and calcium stearoyl-2-lactylates were from Patco Products. Stearoyl-2-lactylic acid was furnished by Glidden-Durkee Division, SCM Corp. Sodium stearyl fumarate was a product of Charles Pfizer & Sons. Succinoylated monoglycerides were from Eastman Kodak, DPI Division. Dioctyl sodium sulfosuccinate was obtained from American Cyanamid Co. The methyl silicone was a Dow Corning product that contained 10% active antifoam material. Phosphatidylethanolamine was obtained from Nutritional Biochemicals Co. and polyoxyethylene sorbitan monostearate from Atlas Powder Co.

RESULTS AND DISCUSSION

Figure 1 is a plot of decrease in iodine value against increase in viscosity for a series of safflower oil samples that had been heated at 180 C for 46 hr. Each point on the curve represents an individual heated sample. The controls containing no additive are grouped at the upper right, indicating extensive polymerization. Those samples containing some of the

TABLE I

Fatty Acid Composition of Heated Samples

Fatty acid	Composition, %[a]			
	Unheated	Control	Me Silicone, 5 ppm	Phosphated MGS, 0.2%
C_{12}	0.1	0.1	0.1	0.1
C_{14}	0.1	0.1	0.1	0.1
C_{16}	7.0	6.3	6.8	6.8
C_{18}	2.2	2.2	2.4	2.6
$C_{18:1}$	13.4	10.6	12.4	12.7
$C_{18:2}$	76.0	50.1	76.2	74.6
C_{20}	0.5	0.3	0.6	0.6
Oxidized[b]	0.7	30.3	1.4	2.5

[a]Internal standard, $C_{28}H_{58}$.
[b]Calculated by difference.

TABLE II

Gas Liquid Chromatography of Heated Samples

Sample	Per cent		
	Triglycerides	Urea nonadducting	Dimers
Unheated	100	1.3	0.1
Control	39.8	27.7	6.2
Methyl silicone, 5 ppm	95.1	6.9	2.2
Phosphated MGS, 0.2%	93.7	8.3	2.6

more effective additives fall near the origin and show only a small change from the unheated oil. From the curve it is evident that oil viscosity increases rapidly during the initial stages of oxidation but levels off as oxidation proceeds.

The additives that compare most favorably with methyl silicone are the sodium salts of phosphated mono- and diglycerides. These surfactants are prepared from liquid, from partially hydrogenated and from fully hydrogenated vegetable oils. All three of these products are active polymerization inhibitors, but the saturated one is the best. Figure 2 is a plot of iodine value vs. time of heating at 180 C. The phosphated derivative at the 0.2% level is seen to be approximately as effective as 5 ppm methyl silicone. Higher levels of up to 1% phosphate ester gave little additional protection. Lower levels were also tested. At 0.1% this additive was still quite effective, but as the level was decreased further it gave less and less protection. At 0.002% only a very small positive effect was observed.

From the above date it is apparent that relatively high levels of phosphated monoglycerides are required to protect safflower oil from polymerization. In terms of resistance to oil color development during heating, this additive functions approximately as well as does the silicone. It is the only additive described in this report that is satisfactory in this respect.

At the termination of the 46 hr heating period, a small amount of insoluble residue was observed in the oils containing added phosphated monoglyceride. This is indicative of relatively low oil solubility. If the solubility of the additive is too high, little stabilization was observed, e.g., with the diacetyltartaric acid esters of mono- and diglycerides, which are quite oil soluble, poor results were obtained (Fig. 3). However, when this material was dispersed in hot distilled water (resulting pH 2.2), neutralized stepwise with sodium hydroxide solution, and subsequently vacuum dried, an improvement in functionality was noted as the pH was raised. Reducing the oil solubility by conversion of the material to its sodium salt seems to improve its effectiveness as a polymerization inhibitor.

This behavior leads us to suggest that these anionic surfactants function in a manner similar to methyl silicone, i.e., a fresh film of additive is forming continuously at the oil-air interface. This film acts as an oxygen barrier to protect the oil. Some support for this mechanism comes from the fact that when the oil is stirred mechanically the protective effect disappears. Presumably stirring disrupts the continuous film which would otherwise form at the surface.

To confirm that a real protective effect is

TABLE III

Protective Indices of Safflower Oil Additives[a]

Additive	%	Protective index
Phosphated MGS	0.02 – 0.2	2.1 – 4.3
Sodium DTAE of MG	0.2	2.2 – 4.4
Stearoyl-2- lactylic acid	0.2	1.3
Sodium stearoyl-2-lactylate	0.1 – 0.2	1.7 – 5.3
Calcium stearoyl-2-lactylate	0.2	0.9
Sodium stearyl fumarate	0.2	3.3
SMG	0.2	1.1
Sodium SMG	0.2	1.6
Dioctyl sodiums sulfosuccinate	0.2	3.0
Sodium sulfoacetate ester of MG	0.2	3.4
Methyl silicone	5 ppm	2.3 – 4.9

[a]46 hr at 180 C.

TABLE IV

Protective Indices of Safflower Oil Additives[a]

Additive	%	Protective index
Sodium stearate	0.1 – 0.2	3.1 – 3.7
Sodium oleate	0.2	3.1
Sodium lauryl sulfate	0.1 – 0.2	1.8 – 2.0
NaH_2PO_4	0.2	0.9
Potassium sorbate	0.2	1.3
Phosphatidylethanolamine	0.1 – 0.2	3.3 – 4.7
Tween 60	0.2	0.9

[a]46 hr at 180 C.

obtained with phosphated monoglycerides, fatty acid composition data was obtained on the heated oils as illustrated in Table I. The heated control had lost a substantial portion of its linoleate, whereas those oils containing added methyl silicone or phosphated monoglycerides had retained substantially all of their polyunsaturates intact. A further analysis of the heated oils is given in Table II. Here we see results of direct gas liquid chromatography on the intact triglycerides. This is further evidence that the control oil had become extenisively polymerized. Methyl silicone showed a slight edge over the phosphated monoglycerides. The fatty acids were recovered from the heated oils, and levels of urea nonadducting material were determined (5,6). Again there was a relatively high level in the heated control and much smaller amounts in the samples containing the additives. TMS derivatives were then prepared from the urea nonadducting fatty acid fractions, and these were chromatographed to determine the levels of dimers in each sample. The results shown in Table II are expressed as the percentage dimer based on the weight of heated oil.

To illustrate the type of separation obtained in this analysis, the actual chromatogram is given in Figure 4. Peak 8 with shoulder 9 represents the oxidative and thermal dimers which emerge together at a retention time close to that of dipalmitin. A small amount of trimer at 11 has a retention time near that of tripalmitin. Peak 1 represents the unoxidized fatty acid TMS derivative, and peak 2 is probably a hydroxy or keto derivative.

A number of other edible anionic surfactants were also found to give a protective effect. Some of them looked quite promising, except that in all cases charring was observed during the heating period. The heated safflower oil samples containing these additives were all significantly darker than the heated controls.

In order to simplify presentation of the data the term "protective index" has been used. Protective index equals Δ iodine value control/Δ iodine value sample tested. A protective index greater than 1.0 indicates a positive effect.

Table III contains data on a series of these additives. The sodium salt of phosphated mono- and diglycerides and the neutralized diacetyltartaric acid esters of mono- and diglycerides are approximately equivalent in their protective indices, but the latter material causes darkening of the oil. Stearoyl-2-lactylic acid gave only a small effect, but its sodium salt was much more functional. The calcium salt was completely ineffective. Perhaps, as suggested earlier, the degree of solubility is an important factor. The sodium salts are evidently good film formers of limited oil solubility. Sodium stearyl fumarate, dioctyl sodium sulfosuccinate and the sodium sulfoacetate esters of mono- and diglycerides were also quite functional. Succinoylated monoglycerides gave a very weak effect which was enhanced significantly by conversion to the sodium salt.

Consideration was given to the possibility that these additives might merely be functioning as trace metal sequestrants. But if this were so, they should give good results in the free acid form. Also tests with the sodium salts were run at ambient and at slightly elevated temperatures with no protection from autoxidation whatever.

It seemed likely that ordinary fatty soaps might behave in a manner similar to these surfactants. This was indeed found to be the case. As shown in Table IV, both sodium stearate and sodium oleate gave good protection. The resulting heated oils had dark colors and soapy flavors. Sodium lauryl sulfate and phosphatidylethanolamine were also found to be functional. The latter material, based on its structural similarity to the phosphated monoglycerides, might be expected to protect the oil. This nitrogen derivative imparts a very dark color and a fishy aroma to the heated oil. By contrast the sodium salt of phosphated mono- and diglycerides appears to have good heat stability, so that the heated oil retains a light

color and a good flavor.

It appears that this property of protecting polyunsaturated oils from oxidative polymerization is common to a number of anionic surfactants. Of those tested, the sodium salt of phosphated mono- and diglycerides is the most promising. But even this additive suffers by comparison to methyl polysiloxane, which is highly functional even at levels below 1 ppm.

REFERENCES

1. Martin, J.B., U.S. Patent 2,634,213 (1953).
2. Sims, R.J., J.A. Fioriti and M.J. Kanuk, JAOCS 49:298 (1972).
3. Waltking, A.E., and H. Zmachinski, Ibid. 47:530 (1970).
4. Fioriti, J.A., N. Buide and R.J. Sims, Ibid. 46:108 (1969).
5. Sahasrabudhe, M.R., and V.R. Bhalerao, Ibid. 40:711 (1963).
6. Johnson, O.C., E. Perkins, M. Sugai and F.A. Kummerow, Ibid. 34:594 (1957).

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Effect of Heated Fat upon Metabolism of 1-^{14}C-Acetate in the Rat[1]

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ABSTRACT

Groups of rats were prefed for 18 weeks with fresh or heated corn oil (~5% nonurea adduct) at 10, 20 and 30% protein levels and at 10% protein + 2% cellulose; 1-^{14}C-sodium acetate was injected intraperitonially into each animal and the radioactivity was measured in expelled CO_2 and also in the lipid fractions of liver. In the groups of rats fed 10% protein, one-half of the administered radioactivity was found 50 min after acetate injection into rats fed fresh oil, whereas 90 min were required to reach the same level in rats fed heated oil. The same trend was found in all the groups of rats receiving different protein levels. The conversion of acetate to CO_2 was significantly higher at a 10% dietary protein level than at the 20 or 30%, but there appeared to be no significant difference in the conversions at the 20 and 30% protein levels. However addition of 2% cellulose to the 10% dietary protein level significantly increased the conversion of the acetate to CO_2 in rats fed heated oil. The livers of animals receiving heated fat diets had a higher lipid content, mainly triglyceride. When the liver lipids from rats fed fresh corn oil were separated by argentation thin layer chromatography, the bulk of the radioactivity was found in the saturated fractions. Monoene, diene and triene fractions from the liver lipid of rats fed heated oil had almost twice the radioactivity of those from fresh oil, suggesting the preferential utilization of acetate in the synthesis of these unsaturated fatty acids.

INTRODUCTION

The nutritional and biological effects of heated fats on laboratory animals have been studied extensively in the past several years (1-4). The results of these studies have shown that severely heated fats exert growth-depressing and other abnormal effects on rats. There is, however, disagreement on the extent of damage that mildly heated fats can cause to laboratory animals when administered as a dietary source (5-8). It has been reported that, when such fats were present in only small quantities and mixed with a balanced nutritional diet, they exhibited no adverse effects (9). The causes of impaired growth and other abnormal effects have been variously attributed to the destruction of essential fatty acids, protein, vitamins and inhibition of enzyme action with an imbalanced diet with severely heated fats (10). However, while not grossly visible, the effects of fats that have not been severely heated on animals maintained under less than optimal nutritional conditions may be considerably more subtle (11). These effects may be exerted at the enzyme level.

The biochemical oxidation of lipids has been studied by following the conversion of radioactive lipid and ^{14}C-acetate to $^{14}CO_2$. Bottino et al. (12) simultaneously fed rats a low fat diet and sodium acetate. After 3 hr, the maximum levels of incorporation of ^{14}C into palmitic and oleic acid were reached. In a study using labeled palmitic acid, Shue et al. (13) observed that the components from heated oils which did not form urea adducts reduced the rate of formation of $^{14}CO_2$ from palmitic acid by 30%. These data suggested that the nonurea-adducting fatty acids of heated oils impaired the oxidation of fatty acids in the weanling rats. Perkins et al. (14) recently studied the absorption in the rat of randomized ^{14}C-labeled corn oil and the methyl esters of the nonvolatile oxidation products of corn oil, and demon-

[1]One of seven papers presented in the symposium "Biological Significance of Autoxidized and Polymerized Oils," JOCS-AOCS Joint Meeting, Los Angeles, April 1972.

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TABLE I

Physical Constants and Fatty Acid Composition of Fresh and Heated Corn Oil

Fatty acid	Corn oil, wt%	
	Fresh	Heated
Palmitic	13.0	14.8
Stearic	2.0	2.2
Oleic	28.1	29.9
Linoleic	56.2	51.2
Arachidic	0.9	0.9
Iodine value	132.9	124.2
Refractive index ($N_D^{25.5^\circ}$)	1.4728	1.4748

TABLE II

Diet Formulation and Feed Design

Group	Protein level, %	Composition of feed, g							
		Oil							
		Fresh	Heated	Casein	Salt mix	Vitamin mix	Choline chloride	Dextrose	Cellulose
1.	10	150	---	100	35	10	1.5	703.5	---
2.	10	---	150	100	35	10	1.5	703.5	---
3.	20	150	---	200	35	10	1.5	603.5	---
4.	20	---	150	200	35	10	1.5	603.5	---
5.	30	150	---	300	35	10	1.5	503.5	---
6.	30	---	150	300	35	10	1.5	503.5	
7.	10	150	---	100	35	10	1.5	683.5	20
8.	10	---	150	100	35	10	1.5	683.5	20

strated that such oxidation products were absorbed and metabolized at a slower rate than fresh corn oil.

In the biosynthesis of fatty acids, chain extention occurs at the carboxyl end of the molecule (15). The process of chain extension by acetyl CoA has been followed by the use of ^{14}C-acetate (16). Following ip injection of ^{14}C-acetate in the rat, incorporation of the acetate into the liver lipids was very rapid and occurred within 30 min after administration. Evans and Norcia reported that 1 hr after injection of ^{14}C-acetate from 0.5 to 1.25% of of the administered dosage of radioactivity was incorporated into the liver lipids (17).

The rate of conversion of acetate to fatty acids is influenced by dietary fat. Levelle and coworkers studied lipid metabolism in rats fed diets containing corn oil and medium chain triglycerides (MCT) (18). Their results showed that MCT depressed plasma and total lipids, and further indicated that the adipose tissue of rats fed MCT converted ^{14}C-acetate to fatty acid at a much faster rate than rats fed corn oil. Hill et al. (19) reported that a pronounced decrease in the capacity of the liver to convert acetate carbon to fatty acid was observed as early as 1 hr after fat was administered to rats prefed a nonfat diet. However 12 hr after feeding fat, the capacity of liver to incorporate acetate carbon into cholesterol increased.

Shue et al. (13) previously observed that components from heated fats reduced the rate of oxidation of palmitate to CO_2. It is probable that such components interfered with the metabolism of natural fatty acids. In order to further determine the nature of these effects, the influence of heated fat upon the metabolism of 1-^{14}C-acetate in the rat was investigated. In the present study, rats were prefed with heated and fresh corn oil with different levels of protein for 18 weeks. Sodium acetate 1-^{14}C was injected intrapertonially into the animals which were then placed in metabolic cages. The appearance of radioactivity in the expired air was monitored, the animals sacrificed, and the radioactivity of the liver lipids determined.

MATERIALS AND METHODS

Corn oil was heated at 190 C (frying temperature) for 132 hr in a stainless steel commercial type deep fat fryer. A stainless steel mechanical stirrer was employed at very low speed to cause equal exposure of the oil to air and to heat with minimum turbulence. At the end of the heating period, the oil was cooled and poured into brown bottles, flushed with nitrogen and kept refrigerated at 0 C until ready for use.

The amount of nonvolatile oxidation products formed during heating of the oil was determined by three different methods: (a) urea adduct formation (20); (b) gas liquid chromatography (GLC); and (c) liquid, liquid partitioning (20). In the first method a sample of the heated oil was converted to methyl esters by transesterification with 2% sulfuric acid in methanol. The methyl esters were mixed with urea and methanol (1:2:4) and the nonurea-adducting fraction (UNA) collected, the solvent evaporated, and the residue weighed. This residue represented 12.1% of the original heated oil and contained ca. 60% linoleic acid (by GLC). The remaining material (4.9%) represented oxidation products.

In the second method, the percentage by weight of nonvolatile material in the esters were determined directly by GLC. Esters were separated using a 6 ft x 1/8 in. stainless steel

TABLE III

Body Weight, Liver Weight and Liver Weight to Body Weight Ratios and Liver Lipid Content of Rats Reared on Different Levels of Protein and Fresh or Heated Corn Oil for 18 Weeks

Groups, % protein-fat[a]	Body wt,[b] g	Liver wt,[b] g	Liver-body,[b] wt%	mg Lipid/g liver
10 – FCO	329	10.3 ± 2.1	3.1	43.5 ± 3.5
10 – HCO	284	11.3 ± 0.7	4.0	76.3 ± 2.1
20 – FCO	430	12.8 ± 0.9	3.0	40.0 ± 1.2
20 – HCO	418	15.2 ± 0.7	3.6	62.8 ± 1.0
30 – FCO	437	13.0 ± 0.9	3.0	40.9 ± 1.1
30 – HCO	440	14.9 ± 0.4	3.4	65.1 ± 0.4
10 + C – FCO	348	10.6 ± 0.5	3.1	42.6 ± 1.0
10 + C – HCO	332	11.5 ± 0.3	3.5	74.7 ± 0.9

[a]FCO = fresh corn oil; HCO = heated corn oil; C = powdered cellulose.
[b]Average of three animals.

column packed with 15% ethylene glycol succinate on Chromosorb W. The nitrogen gas flow was maintained at 40 ml/min, injection port 240 C, detector 260 C and column 180 C. The peaks eluted were measured, and the quantity of the substance was calculated by the triangulation method. Methyl arachidate was used as an internal standard. The degree of polymerization was determined to be 3.5-4%. This method measured the total content of materials that were not eluted from the column under a given set of conditions. The fatty acid composition of both the fresh and heated corn oil is given in Table I. In the third method, the methyl esters of the heated fat were partitioned between hexane and 80% aqueous ethanol. After three partitions, 5.3% of the material remained in the ethanol layer.

Preparation of Laboratory Vitamin Mix

A stock mixture of vitamins was prepared by individually weighing the pure vitamins (Vitamin mix (weights in grams): thiamine HCl, 10.0; vitamin B_2, 2.2; vitamin B_6, 2.2; niacin, 10.0; calcium pantothenate, 6.7; para amino benzoic acid, 2.2; inositol, 11.2; folic acid, 0.4; biotin, 0.045; vitamin B_{12}, 0.03; vitamin C, 2.2; menadione bisulphate, 4.8; vitamin A acetate, 1,980,000 IU; choline chloride, 1.5; vitamins D and E dissolved in hydrogenated fat given 2-3 drops orally once a week.); dextrose was added to make 1 kg. Ten grams of this mixture was added to the diet so as to represent 1% of the total diet.

TABLE IV

Time Required for Elimination of Half the Injected Radioactivity[a] as $^{14}CO_2$ in Rats Fed Heated and Fresh Corn Oil at Various Protein Levels[b]

	Half elimination time, min	
Protein levels, %	Fresh	Heated
10%	50	90
20%	100	110
30%	130	140
10% + 2% Cellulose	60	70

[a]1-^{14}C-acetate.
[b]Average of three animals.

Preparation of Diet

The dry ingredients dextrose, casein, Wesson salt mix, vitamins and choline chloride (Table II) were thoroughly mixed in a mechanical mixer before the fresh or heated oil was added. The feeds were prepared weekly in 1 kg batches, stored in brown bottles, flushed with nitrogen and refrigerated. Vitamins D and E were dissolved in hydrogenated coconut oil and fed 2-3 drops orally to rats once a week.

Feeding Design

Thirty-two male weanling Holtzman rats weighing 50-70 g were divided into two categories, one fed fresh oil and another fed heated oil. They were further subdivided into four groups in each category, on the basis of protein level. Protein was supplied at the 10, 20 and 30% levels. In addition, a group was fed 10% protein and 2% cellulose. Rats were housed in individual wire cages with an abundant supply of water. Fresh diet was given once every 2 days and the animals were weighed each week. The rats were fed for 18 weeks before they were sacrificed.

Intraperitonial Injection of 1-^{14}C Sodium Acetate

At the end of 18 weeks, three animals of each set were weighed, and under mild ether anaesthesia 0.25 ml 1-^{14}C sodium acetate was injected intrapertonially. The animals were immediately placed in all-glass metabolic cages. The syringe was washed thoroughly with the scintillation fluid to determine the amount of

TABLE V

Distribution of Radioactivity among Various Lipid Classes of Liver Lipid from Rats Fed Fresh and Heated Corn Oil at Varying Protein Levels[a]

Lipid class	20% Protein		30% Protein		10% Protein		10% Protein + 2% cellulose	
	F[b]	H[b]	F	H	F	H	F	H
Phospholipid	35.0	34.6	33.6	32.7	34.1	33.2	32.4	33.3
Monoglyceride	14.5	15.5	13.8	14.1	16.3	17.2	15.9	16.3
Diglyceride	13.2	14.4	14.1	15.2	13.7	12.6	12.	12.4
Cholesterol	6.4	5.0	7.3	5.8	8.8	7.4	7.4	8.1
Free fatty acid	18.3	14.8	17.3	15.4	15.4	13.7	16.6	14.8
Triglyceride	10.2	15.4	10.4	15.1	9.1	12.7	9.8	14.1
Cholesterol ester	2.1	0.9	1.7	0.5	2.0	1.8	1.0	0.7

[a]Average of three rats.

[b]F = fresh corn oil; H = heated corn oil.

residual acetate. The actual counts injected were determined by subtracting the syringe washings from the quantity of acetate originally present in the syringe contents.

Collection of Expired Carbon Dioxide

Four all-glass metabolic cages were set up in series. A positive pressure was applied at the bottom of the first cage through a compressed air cylinder tank, previously passed through a series of four tubes containing ethanolamine and finally through a gas tower containing lime water to exclude all carbon dioxide from the compressed air. The filtered air was adjusted to carry the expelled carbon dioxide from each metabolic cage through a series of four drierite tubes to remove moisture and finally bubbled through a gas dispersing tube containing ethanolamine cocktail in order to entrap all radioactive carbon dioxide. The CO_2 collection tube contained 150 ml ethanolamine cocktail. Exactly 5.0 ml cocktail was withdrawn at 10 min intervals and the radioactivity measured using a Beckman LS-250 liquid scintillation counter. The time required to achieve 50% elimination of the administered ^{14}C-acetate as $^{14}CO_2$ was calculated.

Ethanolamine Cocktail

A cocktail containing ethanolamine-ethylene glycol monomethyl ether-toluene 1:8:10 was made fresh every day using redistilled reagent grade ethanolamine (21). The toluene contained premixed 0.3% of PPO + POPOP as fluorescence agent.

Labeled Acetate

The 1-^{14}C-sodium acetate in crystalline form of specific activity 50 mc/mM was obtained from the Nuclear Chicago Co. The radioactive acetate was dissolved in distilled water to give ca. 5 μc/0.25 ml aqueous acetate. Following injection, the rats were kept in the metabolic cage for 3 hr with access to saline water. At the end of this time, the animals were sacrificed, and their livers removed and dried by blotting.

Extraction of Liver Lipids

The separated liver was washed repeatedly in saline water to remove blood and connected tissue. The wet weight of each liver was taken before homogenization in a hand homogenizer. Lipids were extracted with chloroform-methanol (2:1), washed with saline water. The chloroform layer was separated, dried over sodium sulfate and the solvent removed using a rotary evaporator. The isolated lipid was weighed and the radioactivity in the total lipid determined with a Beckman LS-250 Scintillation counter.

Thin Layer Chromatography of Total Liver Lipids

A portion of the total liver lipid, isolated as described above, was converted to its corresponding methyl esters by transesterification with 2% sulphuric acid in methanol. After isolation and drying, the methyl esters of total liver lipids were applied to a 20 cm x 20 cm glass plate coated with a 1 mm thick layer of Silica Gel G impregnated with 12% silver nitrate and a small amount of sodium fluorescein. The plates were developed in a solvent system consisting of Skellysolve-diethyl ether-acetic acid 90:10:2, and viewed under UV light. The liver lipids were separated into four bands. The bands and material at the origin were removed by scraping, extracted and the resultant saturated, monoene, diene, triene + tetraene fractions were counted using the scintillation counter.

RESULTS

A comparison of the fatty acid compositions

TABLE VI

Per Cent Recovery of Radioactivity in Thin Layer Chromatographic Fractions of Lipids from Livers of Rats Fed Fresh and Heated Corn Oil at Various Protein Levels[a]

Total liver lipid methyl ester	Fresh corn oil, % protein				Heated corn oil, % protein			
	10	20	30	10 + C[b]	10	20	30	10 + C[b]
Saturated	72.0	70.6	71.1	73.4	48.7	48.8	51.1	48.5
Monoene	15.4	15.4	14.2	15.3	28.6	23.7	24.1	26.7
Diene	4.9	5.8	6.9	4.1	10.7	10.1	11.0	11.4
Tri- and tetraene	6.1	7.0	6.9	5.7	9.5	16.0	12.6	11.3
Origin	1.1	0.7	0.6	1.0	2.3	1.2	0.8	1.9

[a]Average of three rats.

[b]Diet contains 10% protein + 2% cellulose.

(Table I) of the fresh and heated corn oil used in this study indicated that there was a lowering of the linoleic acid content by ca. 5% caused by the heating. The iodine value was also lowered by 6.6%. These values are in agreement with the percentages of oxidized material as shown by three different methods (4-6%). This oil was therefore comparable to that commonly in use in restaurant deep fryers.

In order to metabolically condition the animals used in the present study to diets containing fresh and heated corn oil, they were fed an adequate level of vitamins and various levels of protein prior to the studies involving ^{14}C-acetate metabolism. After 18 weeks of feeding, final weights of the animals in each group were statistically evaluated. A significant difference in body weight between rats fed fresh and heated oil was found in the case of animals fed 10% protein. No significant differences were observed between the fresh and heated oil fed groups receiving 20 and 30% protein or 10% protein + 2% cellulose.

The relationship between body weights, liver weights and the ratio of liver to body weights is shown in Table III. The livers of the rats fed heated oil at the 20 and 30% protein levels were not significantly heavier than the livers of rats fed fresh oil. The feeding of heated oil caused slightly enlarged livers and a somewhat higher liver to body weight ratio than feeding the corresponding fresh oils.

The times required for the recovery of 50% of the radioactivity as carbon dioxide from expired air, after a single dose of 1-^{14}C-acetate given at the end of the 18 week feeding period, are presented in Table IV. At 50 min after injection of labeled acetate, 50% of the administered radioactivity was recovered in the expired $^{14}CO_2$ from rats fed 10% protein and fresh oil. In the rats receiving heated oil, the time required to recover half of the administered radioactivity was 90 min after injection. At each 10 min collection period, more radioactivity was recovered from the group fed 10% protein and fresh oil than the group fed 10% protein and heated oil. Within the first 10 min, 8.7% of activity was found present in the fresh oil group, and only 2.5% was expired as $^{14}CO_2$ by the group of rats fed heated oil.

A lower percentage of $^{14}CO_2$ was recovered from the rats reared on 20% protein with fresh or heated corn oil than the groups receiving 10% protein. At the first 10 min collection period, 3.9 and 1.7% of the administered radioactivity was recovered from fresh and heated oil fed rats, respectively; but the time required for recovery of half of the radioactivity in these groups occurred 100 and 110 min after injection of acetate with 47.0 and 43.5% recoveries for fresh and heated oil fed rats.

The recovery of the expelled labeled carbon dioxide from the 30% protein fed group (Table IV) did not differ substantially from the group fed 20% protein.

The times required for recovery of half of the administered radioactivity for those rats fed heated oil, 2% cellulose and 10% protein were somewhat higher than those obtained from rats fed a similar diet without cellulose.

Statistical evaluation of the values obtained for recovery of radioactivity in expired CO_2 indicated a pattern of significance similar to that for body weights. There appears to be a very close correlation between the body weights and expelled radioactivity in CO_2, though there is no significant difference in the body weight gained for fresh and heated oil fed rats at all levels of protein except for 10% level; but there appears to be a marked difference in the utilization or conversion of acetate to CO_2, as influenced by the presence of heated fat in the diet. The lower recoveries of total expired $^{14}CO_2$, whenever heated fat was fed, may indicate a preferential utilization of acetate for lipid synthesis in the liver resulting in an

accumulation of lipid in the liver, as indicated in Table III. In all cases, when heated fat was fed, 57-63% more lipid was deposited in the liver.

An examination of the distribution of radioactivity in the lipid classes from livers of animals fed both fresh and heated fat (Table V) indicates that the only difference is in the amount of acetate incorporated into triglyceride. In all cases, ca. 30% more radioactivity was found in the triglyceride fraction from animals fed heated fat. When the total liver lipids were separated according to their degree of unsaturation using argentation chromatography, striking differences were obtained (Table VI). The recovered radioactivity of saturated fatty acids in the saturated fraction of liver lipids of rats fed fresh corn oil assayed ca. 71% regardless of protein level fed, whereas that of the heated fat fed animals assayed 49%. In the cases of the mono-, di-, and the higher unsaturated fatty acid fraction, approximately twice the percentage of radioactivity in these acids was found in the animals fed heated fat as in those receiving fresh corn oil. This would seem to indicate involvement of the heated fat in the enzyme systems responsible for synthesis of unsaturated fatty acids.

The results obtained in the present study indicate that rats fed heated fats containing relatively low levels of oxidation products, while exhibiting no growth differences at higher protein levels, exhibit differences in the metabolism of acetate. The decreased metabolism of acetate effected appears to result in an accumulation of lipid, mainly unsaturated triglyceride in the liver of rats fed the diets containing heated fat.

ACKNOWLEDGMENTS

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REFERENCES

1. Witting, L.A., T. Nishida, O.C. Johnson and F.A. Kummerow, JAOCS 34:421 (1957).
2. Perkins, E.G., J.G. Endres and F.A. Kummerow, Proc. Soc. Exp. Biol. Med. 106:370 (1961).
3. Warner, W.D., P.N. Abell, P.E. Mone, C.E. Poling and E.E. Rice J. Amer. Diet. Ass. 40:422 (1962).
4. Landes, D.R., and J. Miller, Nutr. Reports International 5:37 (1972).
5. Rice, E.E., C.E. Poling, P.E. Mone and W.D. Warner, JAOCS 37:607 (1960).
6. Wilding, M.D., E.E. Rice and K.F. Mattil, Ibid. 40:55 (1963).
7. Alfin-Slater, R.D., S. Anerbach and L. Aftergood, J. Amer. Chem. Soc. 36:639 (1959).
8. Noel, G.A., J.C. Alexander and N.R. Artman, J. Nutr. 93:337 (1967).
9. Keane, K.W., G.A. Jacobson and C.H. Krieger, Ibid. 68:57 (1959).
10. Raju, N.V., M. Narayan Rao and R. Rajagopalan, JAOCS 42:774 (1965).
11. Ottolenghi, A., F. Bernheim and K.M. Wilbur, Arch. Biochem. Biophys. 56:157 (1965).
12. Bottino, N.R., R.E. Anderson and R. Riser, JAOCS 42:1124 (1965).
13. Shue, G.M., C.D. Douglass, D. Firestone, L. Friedman and S.J. Sage, J. Nutr. 94:171 (1968).
14. Perkins, E.G., S.M. Vachha and F.A. Kummerow, Ibid. 100:725 (1970).
15. Barron, E., Biochem. Biophys. Acta 116:425 (1966).
16. Quagliariello, E., C. Landeriscina and P. Coratelli. Biochemia. Biophysca. Acta 164:12 (1968).
17. Evans, J.D., and J.N. Norcia, J. Lipid Res. 5:395 (1964).
18. Leveille, G.A., R.S. Pardini and J.A. Tillotson, Lipids 2:287 (1967).
19. Hill, R., W.W. Webster, J.M. Linazasoro and I.L. Chaikoff, J. Lipid Res. 1:150 (1960).
20. Perkins, E.G., and Van Akkeren, JAOCS 42:782 (1965).
21. Jeffery, H., and N. Alvarez, Anal. Chem. 33:612 (1961).

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Novel Methods for Evaluating Deterioration and Nutritive Value of Oxidized Oil[1]

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ABSTRACT

The peroxide content of an oxidized oil is of limited value in estimating the toxicity of the oil, because some decomposition products of peroxides are also injurious to health. Methods of evaluating the toxicity of deteriorated fats and oils have been investigated. A method based on the mortality of chicken embryos following injection of oil into the yolk sac has been found to be much more sensitive and reproducible than tests based on growth rate of weanling rats or mice fed deteriorated oils. An enzyme activity method using alkaline phosphatase has been tested and gives promise of being very rapid, but additional work is required to evaluate this method and to look for better enzyme systems.

INTRODUCTION

Many papers have been published on the nutritive value and toxicity of autoxidized and polymerized oils (1-7), and in summarizing these reports it can be said that hydroperoxides in autoxidized oils and cyclic monomers in polymerized oils are toxic, although recently Ohfuji and coworkers have proposed that straight chain polymers are toxic (8,9). Most of these investigations have dealt with the isolation and structure determination of the toxic components in polymer fractions.

We have often observed that the nutritive value of oxidized oils decreased, even though polymers could not be detected (10). This stimulated us to reinvestigate the toxicity of hydroperoxides and their fission products, and to examine new methods of evaluating the nutritive value of oxidized oils.

Peroxide Value and Animal Growth Rate

Peroxide values (PV) are most often used to estimate the extent of deterioration of autoxidized oils. Usually oils with high PV show toxic effects. In 1960, Andrews et al. autoxidized soybean oil to PV of 100, 400, 800 and 1200 with the help of $CuCl_2$ and $FeCl_2$ (11), and found that animals fed oils with lower peroxide contents grew normally, that growth rate decreased at intermediate peroxide contents, and that animals fed oil with highest PV died.

We prepared oxidized soybean oil having PV of 10, 110, 180, 380, and rapeseed oil with a PV of 180; animal growth tests were carried out with a basal diet similar to that used by Rice et al. (12). The diet consisted of 43.8 g casein,

[1]One of seven papers presented in the symposium "Biological Significance of Autoxidized and Polymerized Oils," JOCS-AOCS Joint Meeting, Los Angeles, April 1972.

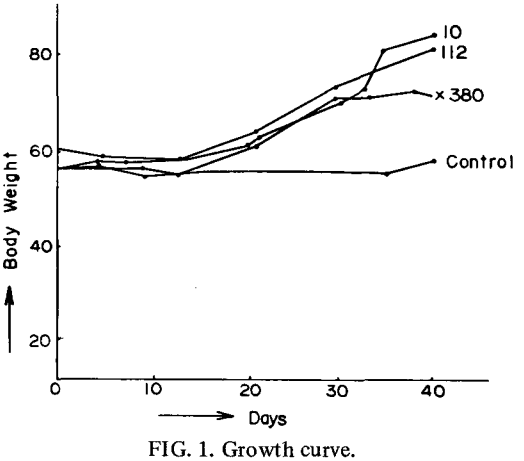

FIG. 1. Growth curve.

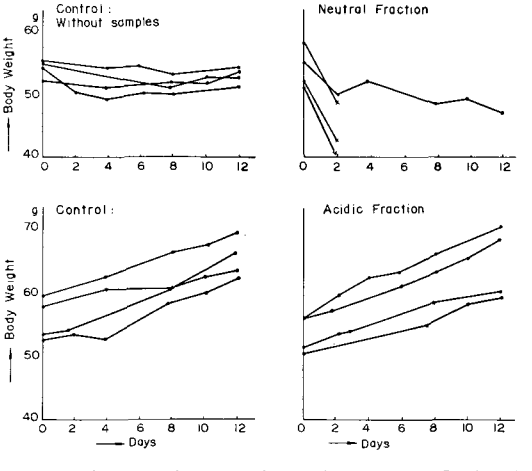

FIG. 2. Growth rates of weaning rats fed 5 g basal diet plus 1 ml neutral or acidic fractions of distillable products.

TABLE I

Preparation of Oxidized Linoleate

Methods	Induction period	Time required, days[a]		
		PV 500	PV 1000	PV 1500
UV irradiation at room temperature	0	2	3	---
In dark at 37 C	14	18	25	31
Ambient light at room temperature	40	54	75	89
Aeration in cold room	104	136	150	---

[a]PV = peroxide value.

30.4 g starch, 13.1 g sucrose, 6.6 g McCollum salt and 2.3 g cellulose. One milliliter of vitamin B mixture contained 1.25 mg thiamin, 1.25 mg riboflavin, 1.25 mg pyridoxin, 6.35 mg niacin, 7.00 mg Ca-pantothenate, 1.60 μg vitamin K, 40.0 μg folic acid, 16.0 μg biotin, 16.0 mg *p*-aminobenzoic acid, 16.0 mg inositol and 160.0 mg choline-chloride in 100 ml solution. Each rat was fed 5 g of diet plus 1 ml of test material per day. Feeding tests showed that animals fed the sample with a PV of 380 gained less weight than those fed the samples with lower PV (Fig. 1).

Toxicity of Fission Products

In these experiments peroxidized oils were heated to destroy the peroxides, but the temperature was controlled to avoid formation of dimers. The heated oils also interfered with growth, indicating that some of the peroxide fission products were toxic.

We collected the volatile decomposition products of heated oils and fractionated them into acidic, carbonyl and neutral fractions (13). Feeding tests carried out with the acidic and neutral fractions showed that the acidic fraction permitted normal growth, but that the neutral fraction caused weight loss and death as shown in Figure 2.

Gas chromatographic analysis of the neutral fraction showed that it was a mixture of hydrocarbons having various carbon chain lengths, and therefore the toxicity of appropriate hydrocarbons was investigated (14). Unex-

TABLE II

Influence of Oxidized Methyl Linoleate on Mice

Amount fed, ml	No. of mice	No. of deaths						
		Days						
		1	2	3	4	5	6	7
UV irradiation								
0.8	6	5	1	—	---	---	---	---
0.6	6	5	0	1	---	---	---	---
0.4	4	0	2	0	---	---	---	---
0.3	4	0	—	---	---	---	---	---
0.2	4	0	---	---	---	---	---	---
Kept at 37 C in dark								
0.8	6	5	1	0	---	---	---	---
0.6	6	1	3	0	---	---	---	---
0.4	4	0	1	1	0	---	---	---
0.3	4	0	---	---	---	---	---	---
0.2	4	0	---	---	---	---	---	---
Kept at room temperature								
0.8	6	3	2	0	—	---	---	---
0.6	6	1	3	0	---	---	---	---
0.4	4	0	—	---	---	---	---	---
0.3	4	0	---	---	---	---	---	---
0.2	4	0	—	---	---	---	---	---
Aeration at -2 to -6 C								
1.0	4	1	2	0	---	---	---	---
0.76	3	1	0	---	---	---	---	---
0.64	5	0	1	---	---	---	---	---
0.50	5	0	—	---	---	---	---	---
0.34	4	0	---	---	---	---	---	---

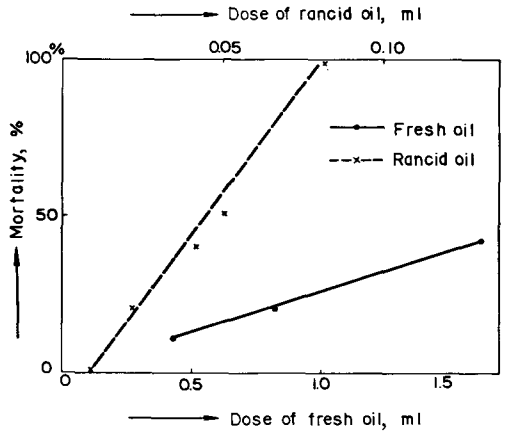

FIG. 3. Dose response curves for fresh and rancid oils by chicken embryo tests.

pectedly, hydrocarbons with less than nine carbon atoms were not toxic, whereas hydrocarbons having more than ten carbon atoms depressed growth. These experiments showed that the toxic components of oxidized oil are complex, and include some of the decomposition products.

Toxicity of Methyl Linoleate Oxidized under Different Conditions

Samples of methyl linoleate were autoxidized by: (a) bulk exposure to air at room temperature under illumination of UV germicidal lamp; (b) exposure to air in bulk at 37 C in the dark; (c) exposure to air in bulk at room temperature; and (d) aeration at -2 to -6 C. Peroxide values in Table I show that these samples autoxidized at widely different rates.

These samples were diluted with fresh linoleate to the same PV level and were fed by stomach tube to mice in various amounts as described in Table II. The results show that, even if the peroxide values of the samples administered were the same, the biological effects were quite different. The toxicity of the materials evidently was related to the rate of decomposition.

TABLE III

Reproducibility of Chicken Embryo Tests[a]

Duration of irradiation,[b] hr	Mortality after 72 hr, %	
	Average[c]	Standard deviation
0	1.6	4.1
200	22.0	4.7
800	94.0	5.5
1000	100.0	0

[a]Yukagaku 20:337 (1971).
[b]Irradiation by germicidal lamp.
[c]Average of six experiments with 10 chick embryos.

Chicken Embryo Test

Recently Miura et al. described a chicken embryo test (15) in which 6-day-old white Leghorn fertile eggs were used to test the toxicity of oxidized oils. Two-tenths milliliter of the sample oil was injected into the yolk sacs of each of 10 eggs, and the injected eggs were incubated at 38 C and 68% relative humidity. The toxicity of the oil was determined by the mortality of the embryos 24, 48 and 72 hr later. Their samples were a mixture of lard and sesame oil (1:1) which had been irradiated by means of a germicidal lamp. Their data (Table III) showed that the reproducibility of this test is good and that toxicity was directly related to the duration of the irradiation.

Figure 3 shows the dose response curves based on mortality of chicken embryos treated with fresh oil or oxidized oil. In both cases, straight line relationships were obtained between dose and mortality. When egg yolk sacs

TABLE IV

Chemical Characteristics and Toxicity of Irradiated Oils

Duration of irradiation	Chemical characteristics				Toxicity		
						Mice	
	PV	CV	AV	IV	Embryo mortality[a]	Mortality[b]	Body wt[c]
0	6	10	0.64	68.6	0	0	↑
150	171	112	1.30	67.2	10	0	↓
300	293	245	2.45	67.6	50	0	↓↓
500	337	492	4.57	58.2	80	0	↓↓↓
800	360	595	9.50	52.3	80	0	↓↓↓
1000	363	650	12.53	47.9	100	80	↓↓↓

[a]Average of two determinations after 72 hr.
[b]Each group consisted of 10 mice.
[c]Body weight at 24 hr after feeding by stomach tube. ↑ = Increase; ↓ = Decrease. PV = peroxide value. CV = carbonyl value and calculations were made by Kumazawa's method. IV = iodine value by Wijs method.

were injected with 0.2 ml fresh oil, the LD_{50} was 1.55 ml. In contrast, the dose-response curve for the oxidized oil was very steep and the LD_{50} was 0.045 ml. In Table IV the chicken embryo test is compared with a mouse toxicity test, using the same rancid samples. Mice of the ddYS strain were fed samples equal to one-twentieth of their body weight by stomach tube. Although mouse mortality occurred only with the most highly oxidized sample, weight loss in the other animals paralleled egg embryo mortality.

Enzyme Inhibition

Ten milligrams of alkaline phosphatase from calf intestinal mucosa was dissolved in 100 ml borate buffer at pH 10.4 and used as a stock solution. Fresh or oxidized linoleic acid was dissolved in propylene glycol at the concentration 3 x 10^{-3} M, added to the enzyme stock solution at a concentration of 10^{-2} M, and incubated at 37 C for 1 hr. Oxidized linoleic acid was prepared under irradiation of an UV lamp for 40 hr (PV 1480). Activities of alkaline phosphatase were determined by the method of King and King. Actual determinations were made by the Yatron alkaline phosphatase determination kit (Ogata Chemical and Medical Research Lab., Tokyo, Japan). Substrate solution was warmed at 37 C for 5 min; then 0.05 ml of samples was added to the 2.0 ml substrate and incubated at 37 C for 15 min. The color-developing reagent (2.0 ml) was added and optical density at 570 mμ was determined. Fresh linoleic acid had no effect on the original alkaline phosphatase activity, whereas oxidized linoleic acid decreased original enzyme activities. Monounsaturated hydrocarbons similar to those isolated from autoxidized soybean oil were also examined. As shown in Table V, the hydrocarbons also depressed alkaline phosphatase activity.

TABLE V

Residual Activities of Alkaline Phosphatase after Exposure to Deteriorated Acids and Hydrocarbons

Materials	%
Fresh linoleic acid	100
Oxidized linoleic acid	40
Decene	84
Undecene	79
Dodecene	81
Tridecene	81

DISCUSSION

Peroxide values are commonly used to evaluate autoxidative deterioration of oils and fats. Normal autoxidation is characterized by an induction period during which little peroxide is formed, followed by an active phase of oxygen uptake and peroxide accumulation. However, when peroxide contents become high, peroxides decompose and the rate of decomposition may become faster than the rate of accumulation. In this case, PV decreases. Moreover peroxides are easily destroyed by heat, and frying fats have low peroxide contents even when they are highly deteriorated. Furthermore, after heating of oxidized oils, the toxicity remains almost the same as that of unheated samples. This suggests that toxic components other than peroxides are present.

As shown in Table II, toxicity is not the same for fats with the same peroxide level. This is probably caused by the fact that the amounts of fission products are not the same.

Many studies on the nutritive value of deteriorated oils and fats have been aimed at the isolation and identification of polymers formed during deterioration. We have studied the toxicity of the fission products and found acrolein to be very toxic, whereas hydrocarbons having more than 10 carbons show weak toxicity. These components are not measured by peroxide determination.

We wanted to find better methods to determine the toxicity of deteriorated oils encountered in daily life. For this purpose, we have introduced the chicken embryo test. The chicken embryo test and animal growth experiments give similar results. The chicken embryo test, however, has the advantage over animal tests of requiring less sample and is much more sensitive and highly reproducible. The chicken embryo test also gives results within 24 hr, in the case of severely oxidized oils.

The enzyme assay test requires a much shorter time and is a good method to estimate the toxicity of a sample. However screening of enzymes to find a better test system is continuing.

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REFERENCES

1. Artman, N.R., in "Advances in Lipid Research," Vol. 7, Edited by R. Paoletti and D. Kritchevsky, Academic Press, New York and London, 1969, p. 245.
2. Crampton, E.W., F.A. Farmer, A.F. Berryhill and L. Wiseblatt, J. Nutr. 43:431 (1951).

3. Crampton, E.W., R.H. Common, F.A. Farmer, F.M. Berryhill and L. Wiseblatt, Ibid. 43:533 (1951).
4. Crampton, E.W., R.H. Common, F.A. Farmer, F.M. Berryhill and L. Wiseblatt, Ibid. 44:177 (1951).
5. Crampton, E.W., R.H. Common, F.A. Farmer, A.F. Wells and D. Crawford, Ibid. 49:333 (1953).
6. Matsuo, N., J. Biochem. (Tokyo) 41:481 (1954).
7. Matsuo, N., Ibid. 41:647 (1954).
8. Ohfuji, T., and T. Kaneda, Yukagaku 21:13 (1972).
9. Ohfuji, T., K. Sakurai and T. Kaneda, Ibid. 21:68 (1972).
10. Akiya, T., Eiyo Shokuryo 15:226 (1962).
11. Andrews, J.S., W.H. Griffith, J.F. Mead and R.A. Stein, J. Nutr. 70:199 (1960).
12. Rice, E.E., W.D. Warner, P.E. Mone and C.E. Poling, Ibid. 61:253 (1957).
13. Akiya, T., Yukagaku 14:347 (1965).
14. Akiya, T., and T. Shimizu, Ibid. 14:520 (1965).
15. Miura, T., M. Tsuchiya and K. Miyaki, Ibid. 20:335 (1971).

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Characterization of Toxic Compound in Thermally Oxidized Oil[1]

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ABSTRACT

In preliminary experiments, soybean oil was heated at 275 C for 12 hr in the presence of N_2 or air. Feeding studies with rats showed that the oil heated in the presence of air, oxidatively polymerized oil, retarded weight gain more than that heated with N_2, thermally polymerized oil. Oxidatively polymerized oil was fractionated with silicic acid column chromatography, and the fraction eluted with ether (fraction III) proved to be most toxic to mice. For chemical studies combined with bioassays, additional oxidatively polymerized oil was prepared by aeration at 185 C for 90 hr, and the product was fractionated by silicic acid column chromatography. The material originally eluted with ether (fraction III) was eluted in stages, and fraction IIIc proved to be most toxic in a mouse bioassay. IR, UV and NMR analyses did not indicate the presence of C-O-O-C or C-O-C linkages or aldehyde groups or aromatic compounds. Fraction IIIc was converted to its methyl esters and molecularly distilled to yield two fractions: one containing unpolymerized fatty acid esters and one giving evidence of more functional groups per molecule and of dimeric material. This "dimeric" fraction was more toxic to mice. IR analysis of this fraction revealed carbonyl groups, and NMR showed several functional groups on alkyl chains. Treatment with sodium borohydride and with hydroiodic acid revealed no C-O-O-C and no C-O-C linkages. Mass spectrography showed peaks at mass 586 and 293; in the reduced fraction a peak also occurred at mass 143, suggestive of cleavage of some of the chains. It is tentatively concluded that a highly toxic material formed in oil heated in air is a dimer of triglyceride molecules.

INTRODUCTION

Crampton and coworkers examined the toxicity of fractions of heat-polymerized linseed oil (1-6). Similar studies with oxidative polymers were carried out by Kaunitz et al. (7) and Perkins and Kummerow (8). In the experiments reported here, we first compared the toxicity of polymerized oils with the ethyl esters prepared from these oils and then attempted to fractionate oxidatively polymerized soybean oil before and after conversion to methyl esters. Bioassays and various analytical techniques have been used in an attempt to correlate chemical structures with toxicity.

[1]One of seven papers presented in the symposium "Biological Significance of Autoxidized and Polymerized Oils," JOCS-AOCS Joint Meeting, Los Angeles, April 1972.

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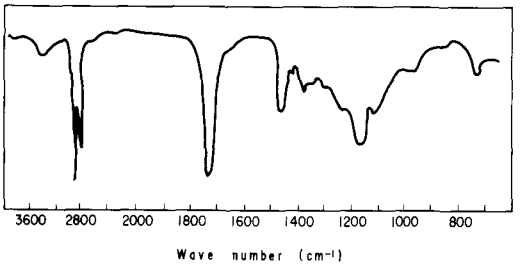


FIG. 1. IR spectrum of fraction IIIc separated from aerated soybean oil heated for 90 hr.

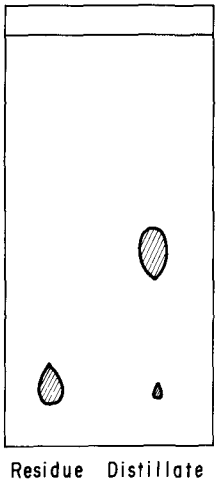


FIG. 2. Thin layer chromatography of distillate and residue separated from fatty acid methyl ester of Fraction IIIc by molecular distillation. Plate: Wakogel B-5; solvent: benzene; indicator: iodine vapor.

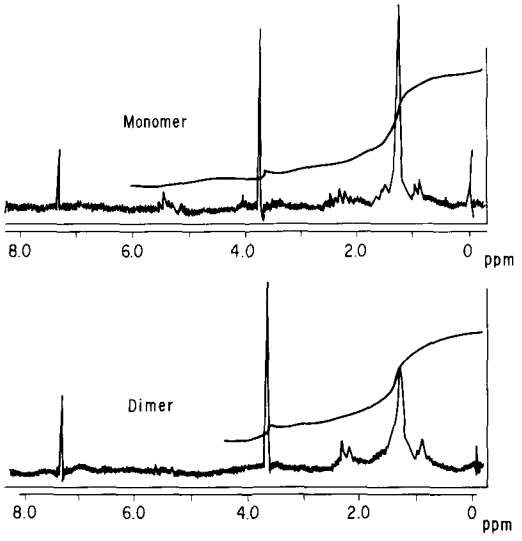


FIG. 3. NMR spectra of monomer fraction (above) and dimer fraction (below) separated from fraction IIIc. Deuterochloroform used as solvent with TMS as reference.

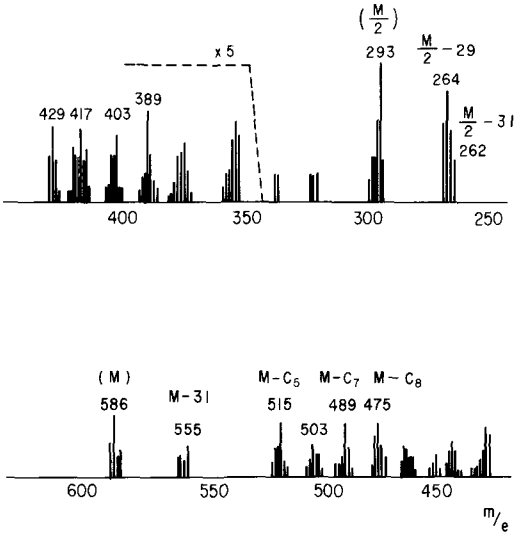


FIG. 4. Mass spectrographic analysis of methyl esters of "dimeric" portion of fraction IIIc.

TABLE I

Analytical Data of Fresh and Polymerized Soybean Oils

Oil characteristics	Fresh oil	TPO[a]	OPO[b]
Acid value	0.12	7.82	3.66
Iodine value, Wijs 60 min	135.0	114.1	115.1
Peroxide value, meq/kg[c]	0.3	7.0	5.2
Saponification value	188.1	186.6	187.8
Carbonyl value, meq/kg[d]	0.99	8.38 (3.37)[e]	12.70 (4.97)[e]
Unsaponifiable matter, %	0.58	2.12	1.46
Viscosity, 30 C, cs[f]	43.8	154.6	190.9
Urea nonadduct, %[g]	(2.86)[e]	(32.55)[e]	(25.55)[e]

[a]Thermally polymerized by heating under nitrogen.
[b]Oxidatively polymerized by heating under air.
[c]Stamm's method (9).
[d]Kumazawa's method (10).
[e]After ethylation.
[f]Ubbelohde's viscometer.
[g]Crampton's method (4).

TABLE II

Influence of Fresh and Polymerized Soybean Oils and of Ethyl Esters of Latter on Rats[a] Fed Purified Diet Containing 20% Oils and Esters for 55 Days

Sample oil[b]	Death rate	Weight gain, g	Digestibility, %	Liver wt/ body wt, %
Fresh	0/4	54.3 ± 0.7[c]	95.3	2.87 ± 0.18[c]
TPO	0/4	20.5 ± 1.0	84.7	4.22 ± 0.22
Ethyl ester of TPO	0/4	24.8 ± 1.3	85.1	3.82 ± 0.20
OPO	1/4	14.2 ± 1.4	82.8	5.14 ± 0.40
Ethyl ester of OPO	1/4	-4.0 ± 2.0	83.5	4.80 ± 0.31

[a]Female rats of Wister strain, 40-49 days old, were used.
[b]TPO = thermally polymerized oil; OPO = oxidatively oxidized oil.
[c]SE of mean.

TABLE III

A. Analytical Data from Sample of Aerated Soybean Oil Fractionated by Silicic Acid Column Chromatography

Effluent solvent	Fraction I (15% IPE in HEX[a])	Fraction II (60% IPE in HEX)	Fraction III (Ethyl ether)
Yield, %	59	31	9
Appearance	Light yellow	Yellow	Red brown, viscous
Acid value	3.1	4.1	6.2
Peroxide value, meq/kg	3.4	16.2	5.4
Hydroxyl value[b]	6.9	27.1	36.3
Carbonyl value, meq/kg	9.6	68.8	84.4
Saponification value	178.4	190.8	223.7
Mean mol wt[c]	904	1505	2056

[a]IPE = isopropyl ether; HEX = *n*-hexane.
[b]Cocks' method (12).
[c]MMW by Hitachi-Perkin Elmer model 115 molecular weight apparatus (vapor pressure equilibrium method).

B. Death Rate of Mice Given Increasing Amounts of Fractions by Stomach Tube[a]

Administrated volume, ml	Administrated oils OPO[b]	Fr. I	Fr. II	Fr. III
Body wt, g, x 1/80	—	—	—	0
Body wt, g, x 1/40	—	—	—	1
Body wt, g, x 1/20	0	0	0	3
Body wt, g, x 1/15	0	0	0	3
Body wt, g, x 1/13	—	1	0	3

[a]Three mice per group.
[b]Oxidatively polymerized oil.

EXPERIMENTAL PROCEDURES AND RESULTS

Comparison of Thermally and Oxidatively Polymerized Soybean Oils

Two batches of 1.8 kg each of soybean oil were heated at 275 C (±5C) for 12 hr. Nitrogen was bubbled through one batch and air through the other at 300-600 cc/min. Part of each batch was converted to its ethyl esters using 0.25% sodium ethoxide in anhydrous ethanol. Table I gives some analytical data obtained for the fresh and polymerized oils. Carbonyl values were considerably elevated in the heated oils, and conversion of the oils to esters resulted in somewhat lower values. The oils and their esters were fed to rats in a diet containing 20% lipid, 51% starch, 20% casein, 4% McCollum salt mixture, 5% brewers' yeast and 10 IU vitamin A per day per rat.

Table II shows that both the thermally (TPO) and oxidatively (OPO) polymerized oils sharply depressed weight gain and that the esters of OPO were even more toxic than the oil, whereas the opposite was true of TPO esters. Liver weights were elevated by the polymerized materials, which has been observed by others (7).

Fractionation of OPO

Oxidatively polymerized soybean oil was prepared as before and fractionated by silicic acid chromatography (11). Fractions of increasing polarity were obtained by successive elution

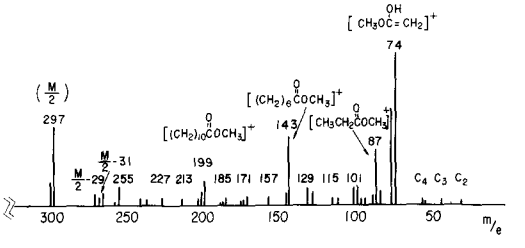


FIG. 5. Mass spectrum of methyl esters of dimer fraction after $NaBH_4$ reduction, and HI-Zn treatment.

TABLE IV

A. Analytical Data from Subfractions of Autoxidized Soybean Oil[a]

Eluting solvent	Fraction II' (IPE[b]/*n*-Hexane) (3:2	III-a (IPE[b])	III-b (IPE[b]/EE[c]) 1:1	(III-c) (EE[c])	III-d (Acetone)
Yield, %	45.8	21.8	18.0	2.6	2.8
Mean mol wt	1280	3660	3280	2390	1980[c]
Carbonyl value, meq/kg	177	450	439	843	---
Peroxide value, meq/kg	7.0	29.2	19.6	15.1	40.2[d]

[a]See text for description of fractions.
[b]Isopropyl ether.
[c]Diethyl ether.
[d]This fraction contained material not soluble in acetone. The molecular weight and the POV were obtained from the acetone-soluble fraction.

B. Death Rate of Mice Fed Increasing Amounts of the Fractions by Stomach Tube

Volume fed, ml	Control[b]	Fraction II'	III-a	III-b	III-c	III-d
Body wt, g, x 1/60	---	--	—	0	3	0
Body wt, g, x 1/40	—	0	0	1	3	0
Body wt, g, x 1/20	0	0	0	1	3	3
Body wt, g, x 1/13	0	0	1	1	3	3
Total dead	0	0	1	3	12	6

[a]Three mice per group.
[b]Methyl ester of olive oil.

TABLE V

A. Properties of Fraction IIIc

Oil characteristics	Fraction IIIc
Mol wt	2050
Conjugated dienes, %	3.0
Conjugated trienes, %	0.0
Peroxide value, meq/kg	11.0
Carbonyl value, meq/kg	870
Hydroxyl value, %	1.46
Epoxide value, %	0.94
Iodine value	74.6
Elementary analysis, %	
C	73.06
H	10.72
O	16.22

B. Calculated Average Content per Molecule of Functional Groups

Component	Per molecule
Total oxygen, 0	20.8
0 of ester	13.9
0 other than ester-0	6.9
0 of carbonyl group	1.8
0 of hydroxy group	1.8
0 of epoxide group	1.2
0 of peroxide group	0.0
Double bond, C=C	6.0
Conjugated diene	0.2

with 15% isopropyl ether in *n*-hexane, 60% isopropyl ether in *n*-hexane, and ethyl ether. Analytical data are summarized in Table IIIA; the least polar fraction I contained unpolymerized triglycerides and fractions II and III, increasing amounts of dimers.

These materials were fed to mice by stomach tube in amounts increasing from one-eightieth to one-thirteenth of their body weight. Table IIIB shows that the most polar fraction (III) contained the most toxic materials.

Concentration of Toxic Materials

Soybean oil was aerated at 185 C (±5C) for 90 hr and was chromatographed on silicic acid as follows: 3 kg silicic acid in hexane was poured into a 10 x 90 cm column and 300 g OPO were used. The first fraction was eluted with 60% isopropyl ether in *n*-hexane and was called II' (see previous experiment); the remaining material was eluted in four fractions with isopropyl ether (IIIa), isopropyl ether-ethyl ether 1:1 (IIIb), ethyl ether (IIIc), and acetone (IIId). Analytical data are given in Table IVA. These fractions were administered to mice by stomach tube in amounts ranging from one-eightieth to one-thirteenth of their body weight (Table IVB). The material eluted with

TABLE VI

A. Analyses on Distillate ("Monomer") and Residue ("Dimer") Fractions of Methyl Esters of Fraction IIIc Obtained by Molecular Distillation

Characteristics	Dimers	Monomers
Mol wt	600	290
Conjugated diene, %	8.6	1.9
Conjugated triene, %	0.0	0.1
Carbonyl value, meq/kg	503	214
Hydroxyl value, %	1.61	0.98
Epoxide value, %	0.40	0.03
Elementary analysis, %		
C	72.21	74.36
H	10.54	12.02
O	17.25	13.62

B. Calculated Content per Molecule of O_2 in Various Functional Groups

Component	Dimers	Monomers
Total oxygen, 0	6.5	2.5
0 of ester	4.0	2.0
0 other than ester	2.5	0.5
0 of carbonyl group	0.3	0.1
0 of hydroxy group	0.6	0.2
0 of epoxide group	0.2	0.0
Conjugated dienes	0.2	0.0

C. Influence of Methyl Esters of Olive Oil and of Fractions Obtained by Molecular Distillation on Survival Rate of Mice Fed Increasing Amounts by Stomach Tube[a]

Volume, ml	Monomers	Dimers	Methyl esters of olive oil
Body wt, g, x 1/80	0	1	0
Body wt, g, x 1/60	0	4	0
Body wt, g, x 1/40	1	4	0
Body wt, g, x 1/20	4	4	0

[a]Four mice per group.

ethyl ether (IIIc) proved to be the most toxic (all dead within 24 hr).

Chemical Studies of Toxic Fraction

Additional amounts of fraction IIIc were prepared, analytical data for which are given in Table VA. These data were used to calculate the values in Table VB. In an attempt to arrive at an average content of certain functional groups per molecule of fraction IIIc, the total amount of oxygen was obtained by elementary analysis and the average oxygen (exclusive of ester bonds) per fatty acid of 18 carbons was calculated. Although it is obvious that the materials forming fraction IIIc represented many molecular species, it could be concluded that, on the average, molecules in this fraction contained two carbonyl groups, two hydroxyl groups and one epoxide group. Figure 1 shows the results of an IR analysis. There was no evidence for the presence of aromatic compounds or of C-O-O-C linkages; the UV spectrum had a peak at 233 mμ, but did not reveal the presence of aromatic compounds. NMR analysis gave no indication of aromatic compounds, aldehydes or ethers.

These observations suggested that the toxic fraction contained dimers of triglycerides formed by dimerization of fatty acids from two different triglycerides. For further analyses, this fraction was converted to its methyl esters using 4% HCl in methanol and the latter were molecularly distilled at 120 C and 5 x 10^{-4} mm Hg. On thin layer chromatography (Figure 2), each subfraction gave a single spot. Table VIA gives the results of chemical analysis, which suggest that the more polar "dimer" fraction contained more conjugated acids and carbonyl groups than did the "monomer" fraction. The "dimer" fractions appeared to

TABLE VII

Fatty Acid Composition (%) of Soybean Oil and of "Monomers" of Fraction IIIc[a]

Carbon no.	Monomer fraction	Soybean oil
7	Trace	—
8	0.2	—
9	Trace	—
10	Trace	—
11	Trace	—
12	Trace	Trace
13	Trace	—
14:0	0.2	0.4
:1	Trace	—
15:0	0.1	—
:1	0.1	—
16:0	20.1	5.4
:1	1.3	2.0
17:0	0.2	—
:1	0.8	—
18:0	6.8	5.7
:1	33.3	27.4
:2	32.7	49.4
:3	2.6	7.6
20>	1.7	2.1

[a]Gas liquid chromatography analyses of methyl ester samples were performed with a Shimazu model GC-4A-PF apparatus under the following conditions: 2 m x 3 mm diameter column containing 15% DEGS on Chromosorb W and operating at 179 C and at 25 ml/mm flow rate of N_2.

contain more functional groups per molecule than did the "monomers" (Table VIB).

These materials were fed to mice by stomach tube (Table VIC), and the "dimers" were more toxic than the monomers.

IR analysis of the "dimers" showed a peak at 1710^{-1} cm indicative of carbonyl groups. NMR spectra of the two fractions are shown in Figure 3. The peaks given by the dimeric fraction suggested the presence of several functional groups on the alkyl chains.

Because oxidative polymers can be linked by C-O-O-C, C-O-C and C-C bonds, the following analyses were undertaken.

C-O-O-C: The dimeric fraction of fraction IIIc was dissolved in methylene chloride-ethanol solution and refluxed with sodium borohydride for 15 hr. This was repeated several times. After reduction, the fraction was acidified with acetic acid, washed with water, and dried. The molecular weight of the treated material was 610, which is very close to the original 600. This was evidence that there were no C-O-O-C linkages.

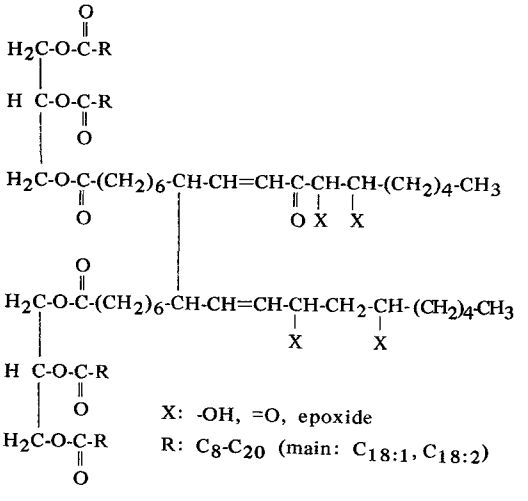
C-O-C: The dimeric fraction was iodinated with hydroiodic acid by the method of Frankel et al. (13), and 0.3 cc of the sample was refluxed with hydroiodic acid at 120 C for 4 hr. Water was added and the reaction mixture was extracted with ether; after evaporation of the ether, the extract was refluxed with zinc dust and glacial acetic acid for 2 hr. Next the zinc was removed by filtration, the filtrate extracted with ether, and the ether removed by evaporation. The molecular weight of the reduced material was 560, which is close enough to the original 600 to suggest the virtual absence of C-O-C and C-O-O-C linkages which would have been opened by the treatment. Thus it would appear that the main linkage is C-C.

Mass spectra are shown in Figures 4 and 5. A peak was noted at mass 586, and there was another pronounced peak at 293. After reduction with hydroiodic acid, peaks were seen at mass 297 and 143, the latter due possibly to cleavage of fatty acids.

Table VII gives the fatty acid compositions of the original soybean oil and of the monomeric fraction. The monomeric fraction contained relatively less linoleate and linolenate.

DISCUSSION

A brief consideration of the analytical results permits some speculation as to the chemical configuration of the dimeric material in fraction IIIc from the oxidatively polymerized soybean oil. The fatty acids in this material contained several functional groups such as carbonyl, hydroxyl and epoxide groups. The content of conjugated dienes was low, but the strong UV absorption at 233 mμ suggested ketones conjugated with double bonds. Although the dimeric fraction undoubtedly contains a large number of different chemical entities, the evidence suggests that a substantial part of the fraction consists of molecules following type:



REFERENCES

1. Crampton, E.W., F.A. Farmer and F.M. Berryhill, J. Nutr. 43:431 (1951).
2. Crampton, E.W., R.H. Common, F.A. Farmer, F.M. Berryhill and L. Wiseblatt, Ibid. 43:533 (1951).
3. Crampton, E.W., R.H. Common, F.A. Farmer, F.M. Berryhill and L. Wiseblatt, Ibid. 44:177 (1951).
4. Crampton, E.W., R.H. Common, F.A. Farmer, A.F. Wells and D. Crawford, Ibid. 49:333 (1953).
5. Crampton, E.W., R.H. Common, E.T. Pritchard and F.A. Farmer, Ibid. 60:13 (1956).
6. Common, R.H., E.W. Crampton, F.A. Farmer and A.S.W. DeFreitas, Ibid. 62:341 (1957).
7. Kaunitz, H., C.A. Slanetz, R.E. Johnson, H.B. Knight, D.H. Saunders and D. Swern, JAOCS 33:630 (1956).
8. Perkins, E.G., and F.A. Kummerow, Ibid. 36:371 (1959).
9. Hamm, D.L., E.G. Hammond, V. Parvanah and H.E. Snyder, Ibid. 42:920 (1965).
10. Kumazawa, H., and T. Oyama, J. Jap. Oil Chem. Soc. 14:167 (1965).
11. Ota, S., N. Iwata and M. Morita, Ibid. 13:210 (1964).
12. Cocks, L.V., and C. Van Red, in "Laboratory Handbook for Oil and Fat Analysis," Academic Press, London, 1966, p.120.
13. Frankel, E.N., C.D. Evans and J.C. Cowan, JAOCS 37:418 (1960).

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Pyruvate Metabolism by Rat Lung in Vitro[1]

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ABSTRACT

Rat lung slices were used to examine the net uptake of pyruvate and the incorporation of specifically labeled pyruvate-^{14}C into CO_2 and lipid fractions. Pyruvate uptake and pyruvate-2-^{14}C incorporation into these fractions was increased by raising the pyruvate concentration in the incubation medium from 1 to 25 mM. The addition of 5 mM glucose to the incubation medium decreased the apparent oxidation of pyruvate-2-^{14}C to CO_2 but increased its incorporation into lung lipids. Glucose did not increase net pyruvate uptake by lung slices. The incorporation of pyruvate-1-^{14}C and pyruvate-3-^{14}C into CO_2 and lung lipids was also examined. The results indicated that a major portion of the pyruvate utilized was oxidized to CO_2 with substantial levels found in the fatty acid moiety of lung phospholipids. The low level of pyruvate conversion to phospholipid glycerol, possibly via the dicarboxylic acid shuttle, is of uncertain physiological significance.

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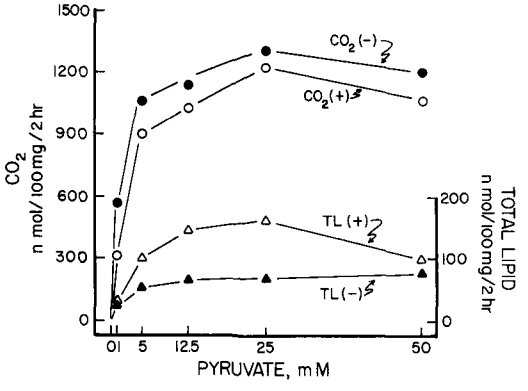

FIG. 1. Pyruvate-2-^{14}C incorporation into CO_2 and total lung lipids (TL) as a function of pyruvate concentration in incubation medium with (+) or without (-) addition of 5 mM glucose. Incubation medium, 2 ml, consisted of indicated level of pyruvate at a constant specific activity of 0.05 μCi/μmol. Individual flasks contained ca. 50-60 mg sliced lung tissue and were incubated in an atmosphere of O_2/CO_2 95:5 for 2 hr. Each point represents mean of six separate determinations.

INTRODUCTION

The existence of a surface active lipid material (surfactant) lining the pulmonary alveoli that is capable of reducing surface forces at low lung volumes has been firmly established (1-3). Pulmonary surfactant contains high levels of phospholipids, predominantly dipalmitoyl phosphatidylcholine (4-6). The rapid rate of phospholipid turnover in mammalian lung (7,8) suggests an active involvement of this tissue in phospholipid synthesis. These findings gain physiological significance when the enormous surface area and the phospholipid content of the lung surfactant layer are considered.

Numerous studies have demonstrated for mammalian lung active synthesis of fatty acids from various precursors (9-22) and, in addition, the capability of utilizing preformed fatty acids for glyceride synthesis (11,13,14,17,23-27). The incorporation into phospholipids of precursors such as phosphate, choline, ethanolamine, methionine and serine have also been investigated (15,16,28-31). These studies have shown that phosphatidylcholine synthesis in lung tissue proceeds by the two well established pathways: (a) the incorporation into lecithin of CDP-choline and D-$\alpha\beta$-diglyceride; and (b) the stepwise methylation, via CH_3-S-adenosyl methionine, of phosphatidylethanolamine. The relative importance of these pathways in lung lecithin biosynthesis appears to vary according to age and animal species.

Previously we reported ATP-citrate lyase activity and the incorporation of citrate carbon into lung fatty acids (21). The presence of low levels of phosphoenolpyruvate carboxylase activity suggested this tissue was capable of converting oxaloacetate to phosphoenolpyruvate and thus of participating in the reversal of the pyruvate kinase step of glycolysis. This possibility was investigated further by examining the incorporation and utilization of pyruvate by lung slices and the distribution of radioactivity in the glycerol moiety of mixed lecithins following incubations with pyruvate 1-^{14}C, 2-^{14}C or 3-^{14}C.

METHODS

Animals

Adult male Long-Evans Hooded rats averaging 400 g body wt were used in the initial experiments on pyruvate-2-^{14}C incorporation

by lung slices. Later studies were with rats of the same strain averaging 300 g body wt. All animals were housed individually in stainless steel cages with raised wire floors and were fed a standard pelleted rat diet (Ralston Purina Co., St. Louis, Mo.). The rats were maintained in a controlled environment; 22 C, 50% relative humidity, and a 12 hr light-dark cycle, respectively. Fasted rats were deprived of food for 48 hr. All rats were offered water ad libitum.

Techniques with Lung Slices

The rats were killed with an ip sodium pentobarbital injection, 30 mg/100 g body wt, and bled via a carotid artery. The lungs were perfused in situ with calcium-free Krebs-Ringer bicarbonate (KRB) buffer, pH 7.4(32), as described previously (19). Lung slices averaging 50-60 mg were incubated for 2 hr in 10 ml reaction flasks fitted with a hanging center well containing a piece of fluted filter paper. The flasks were gassed with O_2/CO_2 95:5 for 5 min, sealed with rubber stoppers, and shaken in a reciprocating water bath (90 strokes per minute) maintained at 37 C. At the end of the incubation period the filter paper was saturated with 0.2 ml hydroxide of hyamine and 0.25 ml 0.4 N H_2SO_4 was added to the incubation medium to liberate CO_2. Shaking was continued for an additional 60 min. The incubation medium (2 ml), used to study pyruvate-2-^{14}C incorporation into CO_2 and lung lipids as a function of pyruvate concentration (1-50 mM pyruvate), contained pyruvate-2-^{14}C at a constant initial specific activity of 0.05 μCi/μmol, KRB buffer and either the presence or absence of 5 mM glucose. The incubation system for pyruvate 1-^{14}C, 2-^{14}C and 3-^{14}C utilization in later studies with fed and fasted rats contained initially 10 mM pyruvate (1 μCi), KRB buffer and the presence or absence of 5 mM glucose.

Isolation of Lipid Fractions

The procedures for extracting, isolating and counting the radioactivity in the various lipid fractions and $^{14}CO_2$ were as described previously (19), with the exception that alkaline hydrolysis of the phospholipid fraction was performed at 70 C for 1 hr.

The isolation of mixed lecithin glycerol radioactivity was accomplished as follows: Portions of total lung lipid were spotted on preparative thin layer plates with commercial egg yolk lecithin (Sigma Chemical Co., St. Louis, Mo.) used as a carrier and as a reference standard. Chromatograms were developed in the solvent system described by Skipski et al. (33), dried at room temperature, and the lipid spots detected by iodine vapor. Areas corresponding to standard lecithin were scraped into centrifuge tubes and eluted with three methanol washes. The samples were concentrated to dryness under nitrogen and saponified with 1.0 ml 3.75% methanolic KOH for 8-10 hr at 60 C. Saponification mixtures were acidified and extracted three times with light petroleum (bp 30-60 C). The samples were again concentrated to dryness under nitrogen and resuspended in 1.0 ml 0.01 M tris, pH 10.4, containing 5 units alkaline phosphatase. This mixture was incubated with shaking for 6-8 hr at 37 C. Hydrolysis was complete as verified by using known concentrations of egg yolk lecithin or L-α-glycerophosphate and determining enzymatically the amount of glycerol produced (34,35). One μmole portions of glycerol were added to the resulting solutions and the ionic constituents removed by passage over microcolumns of Dowex-50 (H^+-form) and Dowex-2 (Cl^--form). Five milliliter fractions containing essentially all the glycerol were collected. One milliliter portions were removed and total glycerol radioactivity counted in 10 ml Bray's scintillation solution (36). Radioactivity in the α-carbons was determined after degrading the remaining glycerol by periodate oxidation and subsequent isolation of the α-carbon formaldehyde as the dimedon derivative (37). Standard amounts of glycerol 1,3-^{14}C and glycerol 2-^{14}C revealed that greater than 95% of α-carbon radioactivity and less than 3% β-carbon radioactivity could be recovered using this procedure. Radioactivity counting was performed in a Nuclear-Chicago scintillation spectrometer, and counting efficiency was determined by the channels-ratio method.

TABLE I

Incorporation of Pyruvate-2-^{14}C into CO_2 and Phospholipids by Rat Lung Slices as a Function of Medium Glucose Concentration

Medium glucose, mM	Pyruvate incorporated, nmol/100 mg/2 hr[a]	
	CO_2	Phospholipids
0	1793.3 ± 92.1[b]	44.6 ± 5.6
5	1641.2 ± 95.4	100.5 ± 11.8
12.5	1389.0 ± 78.1	83.6 ± 15.8
25	1313.3 ± 102.2	76.3 ± 17.9
50	1204.4 ± 72.1	68.8 ± 17.8

[a]Incubation medium, 2 ml, consisted of 20 μmol pyruvate (1 μCi pyruvate-2-^{14}C) and indicated level of glucose in KRB (Ca^{++}-free) buffer, pH 7.4.

[b]Individual values represent means ± SEM of six determinations.

Pyruvate and Glucose Determinations

Experiments designed to determine net up-

TABLE II

Incorporation of Specifically Labeled Pyruvate-^{14}C into CO_2 and Lung Lipids by Fed and Starved Rats

Pyruvate-^{14}C incorporated[a]	Fed ad lib		Starved 48 hr	
	-Glucose	+Glucose	-Glucose	+Glucose
Pyruvate-1-^{14}C				
CO_2	3308.8 ± 335.8[b]	2765.6 ± 263.6	2297.6 ± 270.7	2314.3 ± 161.1
Total lipid	7.4 ± 1.9	12.3 ± 2.1	5.2 ± 1.3	8.0 ± 1.1
Phospholipid	5.1 ± 1.4	8.5 ± 2.3	3.6 ± 0.8	5.2 ± 1.1
Phospholipid fatty acids	<1	<1	<1	<1
Pyruvate-2-^{14}C				
CO_2	1984.2 ± 150.5	1353.8 ± 191.1	1569.3 ± 162.0	1277.3 ± 157.7
Total lipid	98.9 ± 20.3	174.1 ± 47.3	64.0 ± 6.3	133.6 ± 34.9
Phospholipid	60.5 ± 19.6	110.4 ± 32.9	40.0 ± 3.6	61.2 ± 24.4
Phospholipid fatty acids	44.5 ± 7.0	72.0 ± 4.3	27.6 ± 4.3	52.3 ± 14.3
Pyruvate-3-^{14}C				
CO_2	1691.9 ± 153.1	1197.1 ± 155.9	1386.1 ± 121.1	985.2 ± 63.8
Total lipid	127.9 ± 10.9	299.9 ± 15.5	88.7 ± 17.7	118.9 ± 27.9
Phospholipid	80.1 ± 7.5	190.0 ± 40.4	58.8 ± 10.7	76.5 ± 22.3
Phospholipid fatty acids	47.4 ± 7.4	125.3 ± 6.6	40.2 ± 6.8	62.4 ± 10.2

[a]Incubation medium, 2 ml, consisted of 20 μmol pyruvate (1 μCi pyruvate-1-^{14}C, -2-^{14}C or -3-^{14}C) in KRB (Ca^{++}-free) buffer, pH 7.4, with (+) or without (-) the addition of 5 mM glucose. Results are expressed as nmol specifically labeled pyruvate-^{14}C incorporated/100 mg sliced lung/2 hr.

[b]Individual values represent means ± SEM of six determinations.

take of pyruvate and glucose by lung slices were similar to those described for experiments using isotopes. Tissue slices were incubated in the presence or absence of the indicated levels of substrates for 2 hr, after which 1.0 ml incubation medium was placed in 4 ml 6% $HClO_4$. Pyruvate and glucose levels were determined enzymatically by the methods of Bucher et al. (38) and Bergmeyer and Bernt (39), respectively.

Materials

Pyruvate 1-^{14}C, 2-^{14}C and 3-^{14}C were purchased from New England Nuclear Corp., Boston, Mass. Enzymes, cofactors and most biochemicals used in these studies were purchased from Sigma Chemical Co., St. Louis, Mo. All other reagents were of reagent grade quality.

RESULTS AND DISCUSSION

The incorporation by lung slices of pyruvate-2-^{14}C into $^{14}CO_2$ and total lipids as a function of pyruvate concentration in the incubation medium is shown in Figure 1. Increasing the pyruvate concentration to 25 mM increased its incorporation into CO_2 and total lung lipids; however, at a concentration of 50 mM, pyruvate incorporation was reduced markedly in the presence of glucose. The addition of 5 mM glucose to the incubation medium decreased the apparent oxidation of pyruvate-2-^{14}C to CO_2 at all levels of pyruvate. This effect possibly is due to label dilution and would be expected if glucose and pyruvate share common metabolic pathways in lung tissue. Pyruvate-2-^{14}C incorporation into total lung lipids, however, was increased by the addition of glucose to the incubation medium. One explanation for these results has been suggested by Halperin (40-42) and Halperin and Robinson (43), who observed similar results with adipose tissue slices. They postulated that glucose enhances pyruvate incorporation into tissue lipids in vitro by supplying NADPH for the reductive biosynthesis of fatty acids. This explanation appears plausible, since hexose monophosphate pathway activity has been demonstrated for lung tissue (19,44,45). An additional possibility is that glucose may also provide α-glycerophosphate for the esterification of fatty acids, thus effecting greater pyruvate utilization (46). Approximately 70% of the total lung lipid radioactivity was present in the phospholipid fraction as determined by silicic acid column chromatography, with greater than 80% of the phospholipid radioactivity present in the fatty acid moiety following alkaline hydrolysis (data not shown). Low levels of radioactivity originating from pyruvate-2-^{14}C were consistently found in the phospholipid glycerol moiety. The enhanced incorporation of pyruvate-2-^{14}C into lung lipids in the presence of 5 mM glucose was accompanied by increased synthesis of fatty acids.

The possibility that glucose availability limited pyruvate incorporation into lung phospholipids was examined, and the results are presented in Table I. Pyruvate-2-^{14}C oxidation to CO_2 was greatest and its incorporation into phospholipids was lowest when glucose was omitted from the incubation medium, as expected. Raising the glucose concentration above 5 mM did not increase the apparent incorporation of pyruvate into phospholipids. Although it is impossible to compare pyruvate incorporation with glucose incorporation directly from the results of the present studies, phospholipid synthesis from pyruvate in the presence of high levels of glucose was substantial since glucose carbon readily is converted into lung phospholipid fatty acids (14,19,22), in addition to phospholipid glycerol.

The incorporation of specifically labeled pyruvate-^{14}C into CO_2 and lung lipids by fed and fasted rats is shown in Table II. Starvation, in general, decreased the incorporation of pyruvate into these fractions. With the exception of pyruvate-1-^{14}C incorporation into CO_2, glucose addition to the incubation medium decreased the apparent oxidation of pyruvate. These results may be explained in part by isotope dilution effects. Glucose addition to the incubation medium enhanced pyruvate incorporation into lung lipids in both fed and fasted rats. More pyruvate-1-^{14}C was oxidized to CO_2 and essentially none was incorporated into phospholipid fatty acids, as compared with pyruvate-2-^{14}C or pyruvate-3-^{14}C. This is to be expected, since carbon 1 of pyruvate-1-^{14}C is lost as $^{14}CO_2$ during the formation of acetyl-CoA. Pyruvate-2-^{14}C oxidation to $^{14}CO_2$ was greater than that observed for pyruvate-3-^{14}C and was accompanied by a lower level of lipid incorporation. These differences have been explained on the basis of different rates of pyruvate carbon utilization in the tricarboxylic acid cycle (47).

Extensive data have accumulated recently on the incorporation of pyruvate into glyceride-glycerol of adipose tissue (37,48-56). The metabolic pathways by which the reversal of glycolysis occurs have received considerable attention, since adipose tissue apparently lacks the full complement of enzymes that would enable it to undergo gluconeogenesis (57). Theoretically, glycolysis could be reversed directly at the pyruvate kinase step, although this generally is considered thermodynamically unfavorable, or through the abbreviated dicarboxylic shuttle involving pyruvate carboxylase and phosphoenolpyruvate carboxylase. The latter pathway involves the mitochondrial formation of oxaloacetate through pyruvate carboxylation, potential randomization of carbon atoms 1 and 4 and of carbon atoms 2 and 3 with fumarate, and decarboxylation to phosphoenolpyruvate (58,59). This, in effect, is similar to pathways of carbon flow during gluconeogenesis from lactate and certain amino acids in liver and kidney cortex tissue. Most reports have favored this pathway as playing a major role in the reversal of glycolysis in adipose tissue.

TABLE III

Incorporation of Specifically Labeled Pyruvate-^{14}C into α-Carbons of Mixed Lung Lecithin Glycerol

Radioactive label	Per cent label in α-carbons of lecithin glycerol[a]
Pyruvate-1-^{14}C	94 ± 4[b]
Pyruvate-2-^{14}C	43 ± 6
Pyruvate-3-^{14}C	70 ± 7

[a,b]See footnotes to Table II.

Previously we reported phosphoenolpyruvate carboxylase activity (21), and Bottger et al. (60) have reported low levels of pyruvate carboxylase for rat lung. Thus the enzymes necessary for the reversal of glycolysis are present in lung tissue, and these enzymes may act in the metabolic pathways by which pyruvate carbon was converted to lung lecithin glycerol in the present studies. To examine this possibility further we isolated lung lecithin glycerol, following incubations with specifically labeled pyruvate, and determined the radioactivity in the α-carbons. Theoretical considerations for carbon flow from specifically labeled pyruvate-^{14}C have been given by Chakrabarty and Leveille (37).

The results with lung tissue (Table III) show that approximately half of the lecithin glycerol radioactivity from incubations with pyruvate-2-^{14}C was present in the α-carbons. This could not occur if pyruvate was converted to glycerol via a direct reversal of the pyruvate kinase reaction, but would be expected for the pathway involving oxaloacetate and phosphoenolpyruvate with randomization of carbon atoms 2 and 3 with fumarate. An unequal distribution of radioactivity was observed in the α-carbons of glycerol following incubations with pyruvate-2-^{14}C and pyruvate-3-^{14}C. This could occur if (a) the equilibration of oxaloacetate with fumarate was incomplete or (b) partially through a reversal at the pyruvate kinase step. In this respect it is significant to note that not all the glycerol radioactivity following incubation with pyruvate-3-^{14}C was present in the α-carbons. These results suggest that pyruvate was converted in part to lung lecithin glycerol

TABLE IV

Pyruvate and Glucose Uptake by Rat Lung Slices

Addition(s) to incubation medium	Substrate uptake, μmol/100 mg/2 hr[a]	
	Pyruvate	Glucose
Pyruvate (1 mM)	1.89 ± 0.10[b]	—
Pyruvate (10 mM)	5.23 ± 0.67	—
Glucose (5 mM)	—	2.97 ± 0.60
Pyruvate (1 mM) + glucose (5 mM)	1.52 ± 0.13	2.44 ± 0.44
Pyruvate (10 mM) + glucose (5 mM)	4.40 ± 0.68	2.69 ± 0.52

[a]Incubation medium, 2 ml, consisted of indicated levels of substrate(s) in KRB (Ca^{++}-free) buffer, pH 7.4. Control flasks (without added substrate[s]) were incubated simultaneously, and net pyruvate or glucose uptake was determined enzymatically.

[b]Values in table represent means ± SEM of six observations each.

by pathways involving the randomization of pyruvate carbons 2 and 3, presumably via the dicarboxylic acid shuttle. Additional evidence in support of these results was obtained from consideration of the following data (unpublished results, R.W. Scholz): (a) The addition of oxaloacetate to the incubation medium reduced significantly the incorporation of pyruvate-2-^{14}C into lung phospholipids; and (b) low levels of NaH $^{14}CO_3$ radioactivity were recovered in phospholipid glycerol.

The physiological significance of these results with lung tissue is uncertain at present. We have been unable to detect significant activities of glucose-6-phosphatase or fructose-1,6-diphosphatase in rat lung, which would suggest that this tissue is unable to carry out gluconeogenesis. In adipose tissue the conversion of pyruvate to glyceride-glycerol has been considered from a standpoint of providing α-glycerophosphate for re-esterification of fatty acids (53,55,56), possibly when glucose availability is limiting (50,51). Studies concerned with a quantitative estimation of pyruvate incorporation into lung lipid glycerol have not been undertaken, however, and to assign a similar role in this tissue would be speculative. The low levels of pyruvate carboxylase (60) and phosphoenolpyruvate carboxylase (21) in rat lung casts doubt on the physiological significance of pyruvate incorporation into phospholipid glycerol. The heterogeneous cell population with divergent metabolic activities (61) makes it even more difficult to assign to mammalian lung a metabolic function characteristic of a particular cell-type.

It was of interest to determine net uptake or utilization of pyruvate and glucose in these studies, since isotope incorporation could arise through exchange reactions, in addition to net uptake. The results of these experiments are presented in Table IV. The data show that pyruvate and glucose both were metabolized actively by lung tissue slices. Pyruvate utilization was enhanced by increasing the incubation medium concentration from 1 to 10 mM. These results agree favorably with the results illustrated in Figure 1, which show that pyruvate-^{14}C incorporation into CO_2 and lung lipids was increased as the concentration of pyruvate in the incubation medium was increased. Net pyruvate uptake was not increased by including 5 mM glucose in the incubation medium. These results suggest that the glucose effect in vitro on increased pyruvate-^{14}C incorporation into lung lipids is mediated through an altered metabolism of pyruvate, rather than an enhanced uptake. These data, together with the isotope data, indicate that pyruvate is metabolized actively by lung tissue. A major portion of pyruvate is oxidized to CO_2 with substantial levels found in the fatty acid moiety of lung phospholipids. The low level of pyruvate conversion to phospholipid glycerol is of uncertain physiological significance.

REFERENCES

1. Pattle, R.E., Nature 175:1125 (1955).
2. Clements, J.A., Proc. Soc. Exp. Biol. Med. 95:170 (1957).
3. Mead, J.F., Physiol. Rev. 41:281 (1961).
4. Klaus, M.H., J.A. Clements and R.J. Havel, Proc. Nat. Acad. Sci. U.S. 47:1858 (1961).
5. Buckingham, S., Amer. J. Dis. Child. 102:521 (1961).
6. Brown, E.S., Amer. J. Physiol. 207:402 (1964).
7. Tierney, D.F., J.A. Clements and H.J. Trahan, Ibid. 213:671 (1967).
8. Abrams, M.E., and A. Wigglesworth, Biochem. J. 111:21 (1968).
9. Popjak, G., and M. Beeckmans, Ibid. 47:233 (1950).
10. Lands, W.E.M., J. Biol. Chem. 231:883 (1958).
11. Felts, J.M., Med. Thorac. 22:89 (1965).
12. Nasr, K., and H.O. Heinemann, Amer. J. Physiol. 208:118 (1965).
13. Buckingham, S., H.O. Heinemann, S.C. Sommers and W.F. McNary, Amer. J. Pathol. 48:1077 (1966).

14. Salisbury-Murphy, S., D. Rubinstein and J.C. Beck, Amer. J. Physiol. 211:988 (1966).
15. Chida, N., and F.H. Adams, J. Lipid Res. 8:335 (1967).
16. Spitzer, H.L., K. Morrison and J.R. Norman, Biochim. Biophys. Acta 152:552 (1968).
17. Wolfe, B.M.J., B. Anhalt, J.C. Beck and D. Rubinstein, Can. J. Biochem. 48:170 (1970).
18. Mims, L.C., and P. Zee, Biol. Neonate 18:356 (1971).
19. Scholz, R.W., and R.A. Rhoades, Biochem. J. 124:257 (1971).
20. Schiller, H., and K. Bensch, J. Lipid Res. 12:248 (1971).
21. Scholz, R.W., Biochem. J. 126:1219 (1972).
22. Wang, M.C., and H.C. Meng, Lipids 7:207 (1972).
23. Heinemann, H.O., Amer. J. Physiol. 201:607 (1961).
24. Felts, J.M., Physiologist 5:139 (1962).
25. Naimark, A., J. Appl. Physiol. 21:1292 (1966).
26. Harlan, W.R., Jr., S.I. Said and C.M. Banerjee, Amer. Rev. Resp. Dis. 94:938 (1966).
27. Gluck, L., M.V. Kulovich, A.I. Eidelman and A.F. Khazin, Pediat. Res. 6:81 (1972).
28. Weinhold, P.A., and C.A. Villee, Biochim. Biophys. Acta 160:540 (1965).
29. Gluck, L., M. Sribney and M.V. Kulovich, Pediat. Res. 1:247 (1967).
30. Weinhold, P.A., J. Lipid Res. 9:262 (1968).
31. DiAugustine, R.P., Biochem. Biophys. Res. Commun. 43:311 (1971).
32. Umbreit, W.W., R.H. Burris and J.F. Stauffer, "Manometric Techniques," Burgess Publishing Co., Minneapolis, Minn., 1957, p. 149.
33. Skipski, V.P., R.F. Peterson and M. Barclay, Biochem. J. 90:374 (1964).
34. Wieland, O., "Methods of Enzymatic Analysis," Edited by H.U. Bergmeyer, Academic Press, New York, 1965, p. 211.
35. Larsen, J.A., Acta Physiol. Scand. 57:224 (1963).
36. Bray, G.A., Anal. Biochem. 1:279 (1960).
37. Chakrabarty, K., and G.A. Leveille, Arch. Biochem. Biophys. 125:259 (1968).
38. Bucher, T., R. Czok, W. Lamprecht and E. Latzko, "Methods of Enzymatic Analysis," Edited by H.U. Bergmeyer, Academic Press, New York, 1965, p. 253.
39. Bergmeyer, H.U., and E. Bernt, Ibid., Edited by H.U. Bergmeyer, Academic Press, New York, 1965, p. 123.
40. Halperin, M.L., Can. J. Biochem. 48:1228 (1970).
41. Halperin, M.L., Ibid. 49:736 (1971).
42. Halperin, M.L., Biochem. J. 124:615 (1971).
43. Halperin, M.L., and B.H. Robinson, Ibid. 116:235 (1970).
44. Glock, G.E., and P. McLean, Ibid. 56:171 (1953).
45. Tyler, W.S., and G.E. Pearce, Thorax 20:149 (1965).
46. Cahill, G.F., B. Leboeuf and A.E. Renold, Amer. J. Clin. Nutr. 8:733 (1960).
47. Weinman, E.O., E.H. Strisower and I.L. Chaikoff, Physiol. Rev. 37:252 (1957).
48. Christophe, J., B. Jeanrenaud, J. Mayer and A.E. Renold, J. Biol. Chem. 236:648 (1961).
49. Levielle, G.A., Life Sci. 6:803 (1967).
50. Leveille, G.A., Can. J. Physiol. Pharmacol. 45:201 (1967).
51. Ballard, F.W., R.W. Hanson and G.A. Leveille, J. Biol. Chem. 242:2746 (1967).
52. Kneer, P., and E.G. Ball, Ibid. 243:2863 (1968).
53. Reshef, L.R., R.W. Hanson and F.J. Ballard, Ibid. 244:1994 (1969).
54. Schmidt, K., and J. Katz, Ibid. 244:2125 (1969).
55. Gorin, E., Z. Tal-Or and E. Shafir, Eur. J. Biochem. 8:370 (1969).
56. Reshef, L., R.W. Hanson and F.J. Ballard, J. Biol. Chem. 245:5979 (1970).
57. Weber, G., H.J. Hird, N.B. Stamm and D.S. Wagle, "Handbook of Physiology," Section 5, Edited by A.E. Renold and G.F. Cahill, Jr., American Physiological Society, Washington, D.C., 1965, p. 225.
58. Utter, M.F., D.B. Keech and M.C. Scrutton, "Advances in Enzyme Regulation," Edited by G. Weber, Pergamon Press, New York, 1964, p. 49.
59. Haynes, R.C., Jr., J. Biol. Chem. 240:4103 (1965).
60. Bottger, I., O. Wieland, D. Brdiczka and D. Pette, Eur. J. Biochem. 8:113 (1969).
61. Heinemann, H.O., and A.P. Fishman, Physiol. Rev. 49:1 (1969).

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LETTER TO THE EDITOR

Modification by Excessive Heat of Glyceryl Phosphoryl Ethanolamine on Phenol-Containing Paper Chromatograms

Sir: Among the most powerful and widely used methods for quantitation of individual phospholipids is the procedure for successive chemical hydrolyses originally described by Dawson and coworkers (Biochem. J. 75:45 [1960]; 84:497 [1962]) and later modified by Wells and Dittmer (Biochemistry 5:3405 [1966]). We wish to report the production of an artifact that can be formed during paper chromatography and electrophoresis of water-soluble glycerylphosphate esters according to Dawson (in "Lipid Chromatographic Analysis," Vol. 1, Edited by G.V. Marinetti, Marcel Dekker, Inc., New York, 1967, Chapter 4), if the instructions are not properly followed.

For determination of phospholipid components in a mixture, we have adopted a procedure that combines aspects of both methods cited above. Specifically, we carry out the mild alkaline degradation on phospholipid containing 100-300 μg P for 10 min according to Wells and Dittmer, since their conditions were stated to result in negligible formation of cyclic glycerophosphate from phosphatidyl choline and phosphatidyl inositol. The alkaline reaction mixture is then neutralized with 0.2 ml ethyl formate, taken to dryness, and the residue partitioned between water-rich and chloroform-rich phases as described by Dawson. Descending paper chromatography and electrophoresis of an aliquot of the upper phase are then carried out according to Dawson, except that Whatman no. 43 paper is used, and the chromatography solvent consists of liquid phenol (Fisher Scientific Co.)-water-glacial acetic acid-ethanol (79:21:10:12 v/v).

In the course of applying this technique to analysis of phospholipids in tumors induced by injection of chemically transformed astrocytes into guinea pigs, we repeatedly obtained low values for phosphatidyl ethanolamine-P as a per cent of total lipid P. In addition, following two dimensional separations on paper, we observed P-containing spots which were approximately in the position described by Dawson (in "Lipid Chromatographic Analysis," Vol. 1, 1967) for glyceryl phosphoryl methanol and cyclic glycerophosphate.

Analysis of phospholipids in a rat liver total lipid extract prepared according to Folch et al. (J. Biol. Chem. 226:497 [1957]) gave values for alkali-labile phospholipids similar to data in the literature, with the exception of phosphatidyl ethanolamine which was markedly lower. However, if the amounts of P in the two additional spots were added to the P found in glyceryl phosphoryl ethanolamine, the expected values were obtained.

The conclusion that glyceryl phosphoryl ethanolamine was undergoing modification was confirmed by determination of the water-soluble, mild alkaline degradation products of several commercially obtained phosphatidyl ethanolamines including synthetic dipalmitoyl glyceryl phosphoryl ethanolamine (Serdary Research Labs., London, Ontario) and phosphatidyl ethanolamine from bacterial and bovine sources (Applied Science Labs., State College, Pa., and Supelco, Bellefonte, Pa.). The bovine phosphatidyl ethanolamine consistently gave a low yield of alkali-labile product, presumably because of the presence of plasmalogen. On hydrolysis, all of these preparations yielded as little as 50% glyceryl phosphoryl ethanolamine with the remainder of the P distributed between the two spots mentioned above. A trace of glycerophosphate was usually also seen. Unexpectedly, the same products were also obtained when phosphatidyl ethanolamine was hydrolyzed according to the conditions of Dawson without modification. In contrast, phosphatidyl choline (Analabs, Inc., North Haven, Conn.) gave, as expected, only glyceryl phosphoryl choline.

A systematic check indicated that changing either the reagents used in the mild alkaline degradation or the electrophoresis buffer did not abolish the phenomenon. Moreover, when methanolysis of phosphatidyl ethanolamine was carried out in [^{14}C]-methanol, the spot which migrated to the position of glyceryl phosphoryl methanol was unlabeled.

The cause of the degradation was eventually traced to the drying procedure employed fol-

lowing chromatography in the phenol-containing solvent. Papers were dried at 120-140 C for 1 hr. Under these conditions a brown spot appears, which overlaps glyceryl phosphoryl ethanolamine and seems to be caused by the interaction of formate with the solvent. If the papers were dried at 75 C (Dawson, in "Lipid Chromatographic Analysis," Vol. 1, 1967, recommends 80 C) for 60-90 min, no brown material was observed, and after electrophoresis glyceryl phosphoryl ethanolamine was the only detectable P-containing spot.

Thus, at a temperature greater than 80 C and in the presence of sodium formate and the phenol-containing solvent, a substantial fraction of glyceryl phosphoryl ethanolamine is modified to produce several anionic P-containing species, one of which is probably cyclic glycerophosphate. These findings emphasize the necessity of avoiding too high a temperature to remove phenol from paper chromatograms in separations of water-soluble phosphate esters derived from phospholipids by alkaline hydrolysis.

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Lipase from Egg of Southern Corn Rootworm[1]

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ABSTRACT

The lipolytic activity in homogenates and aqueous extracts of acetone powders of eggs of the southern corn rootworm (*Diabrotica undecimpunctata howardi* Barber) was studied. The general properties were determined using as substrate olive oil in an emulsion stabilized by gum arabic. Bovine serum albumin or Triton X-100 were required in the assay system; they protected the enzyme from spontaneous denaturation. The lipase had activity optima at pH 7 and at 45 C. The preparation was inactive towards triacetin, and activity increased in the series tripropionin < tributyrin < trihexanoin < trioctanoin < tridecanoin. Activity towards triolein was slightly lower than towards tridecanoin. Triolein was hydrolyzed to oleic acid and glycerol with no marked accumulation, even transient, of partial glycerides.

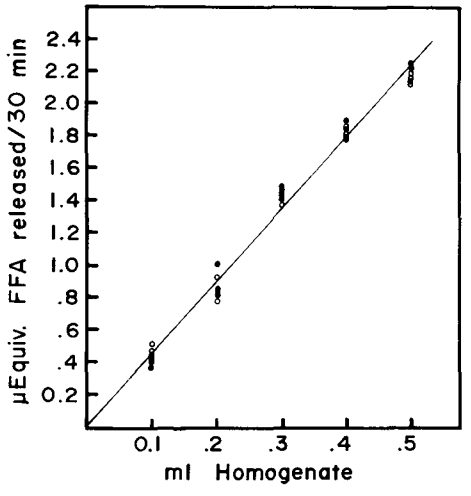

FIG. 1. Effect of homogenate concentration on appearance of free fatty acids. Twenty-five southern corn rootworm eggs were homogenized in 7.5 ml tris-maleate buffer (0.2 M, pH 7.0). Homogenate was added to remaining components of standard assay medium in volumes indicated on abscissa; tris-maleate buffer (0.2 M, pH 7) was added to bring the final assay volume to 1.3 ml. Results represent data from two independently prepared homogenates; triplicate measurements for each homogenate are presented separately (open and closed circles) to illustrate precision of method. Other conditions are as described in Materials and Methods.

INTRODUCTION

Triglycerides are well established as a primary energy source for embryogenesis in insects (1,2). Therefore lipase (E.C. 3.1.1.3; glycerol ester hydrolase) must play a key role in the mobilization of energy. Indeed Fodor (3) demonstrated lipase in an insect egg, and several authors have ascribed to lipase the hydrolysis of short chain triglycerides by insect egg homogenates (4-6). However no insect egg has yet been extensively characterized for its lipolytic activity. Such a characterization is particularly needed because of early difficulties in defining lipase (7) and the diversity of conditions needed for the assay of lipolysis (8). Thus, as part of a study on the mechanisms of regulation of lipolysis in an insect egg, we have prepared this report which describes the general properties of the lipase activity of eggs of the southern corn rootworm, *Diabrotica undecimpunctata howardi* Barber.

MATERIALS AND METHODS

The enzyme source was eggs (0-2 days old) of the southern corn rootworm (SCR) from a laboratory colony maintained by the method of Howe and George (9). Homogenates were prepared immediately before an experiment with

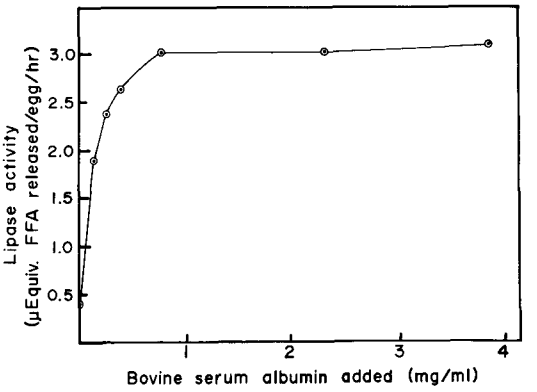

FIG. 2. Lipolysis in presence of varying concentrations of bovine serum albumin (BSA). Enzyme source was whole egg homogenates. The BSA solution normally present in standard assay medium was replaced with requisite volume of water or BSA solution. Highest BSA concentration shown (3.85 mg/ml) is amount present in standard reaction medium. Each point is mean of duplicate measurements. Other conditions are as described in Materials and Methods.

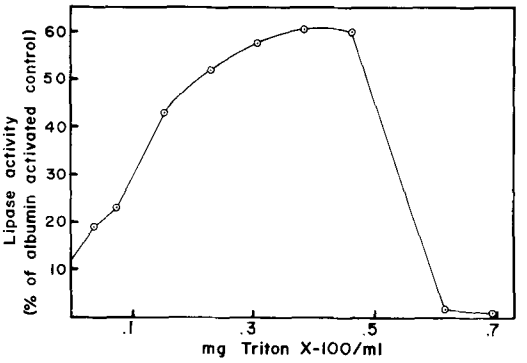

FIG. 3. Lipolysis in presence of varying concentrations of Triton X-100. Enzyme source was whole egg homogenate. Bovine serum albumin (5 mg) was present only in a control series which represented 100% activity; others contained indicated amount of Triton X-100 added in 0.1 ml H_2O. Other conditions are as described in Materials and Methods.

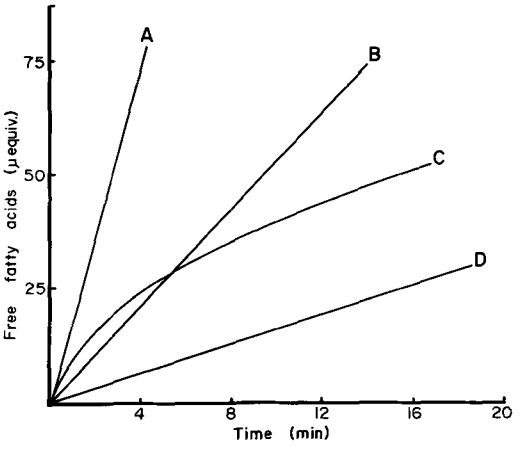

FIG. 4. Time course of lipolysis in presence and absence of bovine serum albumin (BSA). Lines shown were traced from pH-stat recorder curves and rotated 90° clockwise to match conventional format. Only pertinent sections of each experiment have been reproduced and all cases have been adjusted to time zero. See Materials and Methods for composition of basic reaction medium. Trace A: enzyme (acetone powder extract) and BSA (5 mg) present. Trace B: BSA added (5 mg in 0.1 ml H_2O) 1 min after adding enzyme to otherwise complete assay mixture. Trace C: enzyme present, no BSA. Trace D: no enzyme added, and therefore it reflects acid produced by nonenzymic means.

an ice-cold all-glass tissue grinder (25 eggs/3 ml 0.001 M tris-maleate, pH 7.0) and held at 0 C, under which condition the lipase is stable for several hours. For some experiments we used the aqueous extract of acetone powder of SCR eggs prepared as previously described (10). It provided a convenient, stable source of the lipase activity. The two different preparative methods yielded identical lipase zymograms when subjected to polyacrylamide gel electrophoresis (unpublished observations).

Sources of the various triglyceride substrates were: triacetin, Sigma Chemical Company; tributyrin, Fisher Scientific Company; tripropionin, trihexanoin, trioctanoin and tridecanoin, Eastman Organic Chemicals; triolein (A Grade), Calbiochem; olive oil, local commercial sources and Sigma Chemical Company. Free fatty acids (FFA) that contaminated the olive oil were removed by extraction against 3 volumes 0.05 M sodium borate, pH 10.5. The triglycerides were purified with an alumina column by the method of Jensen et al. (11) and purity determined using the thin layer chromatographic method of Guss (12).

Bovine serum albumin (BSA) (fatty acid-free) and Triton X-100 were obtained from Sigma Chemical Company.

For most experiments the enzyme reaction medium and the FFA assay procedure were from a colorimetric procedure described previously (13). Triplicate assays were run at 30 C for 30 min. The experiments relating fatty acyl chain length to activity were done titrimetrically. Titrimetric assays at pH 7 were done using a manual method (14). The assay mixture contained 0.3 ml 0.075 M $CaCl_2$, 0.3 ml 1 M NaCl, 6 ml tris-maleate (0.001 M, pH 7), 6 ml substrate emulsion (15), 5 mg BSA and 0.3 ml enzyme extract (which contained 0.105 mg protein/ml). Titrimetric assays at pH 9 were done using a Radiometer automatic pH-stat equipped with a 0.25 ml syringe and with 0.005 N NaOH as titrant. The assay mixture for this system was 0.5 ml of enzyme source and 9.5 ml of the following mixture: 50 ml 0.5 M $CaCl_2$, 20 ml substrate emulsion (same as for manual titration), 10 ml 0.001 M tris-maleate, 10 ml 1.0 M NaCl, 40 ml H_2O and 50 mg BSA. These titrimetric systems yielded activity that was linear with time for at least 5 min. All titrimetric assays were done under a steady flow of nitrogen. Data are corrected for acid produced by nonenzymic means. Assays were done in triplicate. Methods for studying the enzymatic hydrolysis of radioactive triolein are described by Krysan and Guss (16).

The assay media used were modifications of that used by Mahadevan et al. (15), which contained added $CaCl_2$. We subsequently learned that gum arabic contains Ca^{++} in large quantities (unpublished observations), and then determined that the added Ca^{++} had no effect on activity. We continued to add $CaCl_2$, however, to maintain a constant medium throughout the study.

RESULTS AND DISCUSSION

The experiments described, unless otherwise

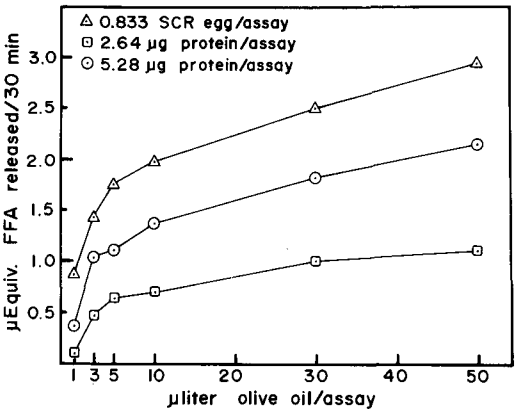

FIG. 5. Effect of olive oil concentration on southern corn rootworm egg lipase activity. Lipase activity was from whole egg homogenates (0.833 eggs per assay, -△-) and extracts of acetone powder of southern corn rootworm eggs (2.64 µg protein/assay, -⊡-; 5.28 µg protein/assay, -⊙-). Substrate concentration was varied by adding requisite amounts of substrate emulsion and emulsifying medium in which water was substituted for olive oil. See Materials and Methods for other assay details.

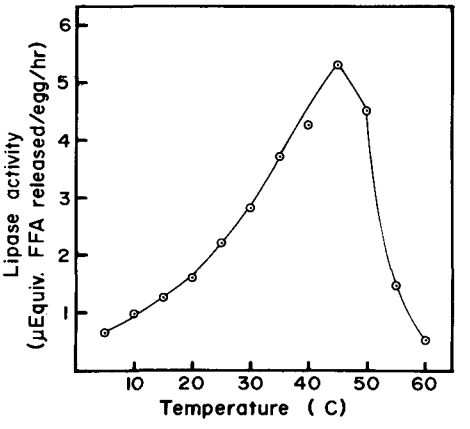

FIG. 6. Effect of temperature on southern corn rootworm egg lipase. Enzyme source was whole homogenate of southern corn rootworm eggs. See Materials and Methods for other details.

noted, were done using the colorimetric method. In a previous report (13) we demonstrated that the activity of the SCR egg lipase in our colorimetric system was linear as a function of time for at least the first 64 min of reaction. The relationship between lipase activity and the concentration of SCR egg homogenate is illustrated in Figure 1.

BSA and Triton X-100 greatly enhanced lipase activity; the relationship between these effectors and lipase activity is illustrated in Figures 2 and 3. We evaluated the mechanism of activation of the enzyme by BSA with the automatic pH-stat, which permitted continuous monitoring of the activity. Results from these experiments, presented in Figure 4 as pH-stat recorder traces, can be summarized as follows. In the absence of BSA, activity declined rapidly with time. In the presence of BSA, activity was linear with time. The addition of BSA 1 min after the onset of the reaction stopped the decline in activity but did not reverse the loss in activity that had already occurred. Many factors, including surfactants and BSA, have been reported to enhance lipase activity by several mechanisms including FFA sequesteration, interface alteration and protection from denaturation (7,8,17). From the series of experiments summarized in Figure 4, we conclude that BSA enhances activity by preventing denaturation of the SCR egg lipase. Triton X-100 similarly protects the enzyme, but the effective concentration range was quite narrow (Fig. 3), and therefore we used BSA as the protective agent in our assay system. In another study we found that the lipase of SCR eggs, when partially purified by electrophoresis on acrylamide gel, was unstable in aqueous solution and could be stabilized by BSA or Triton X-100 (10).

The effect of the concentration of substrate on lipase activity is illustrated in Figure 5. In the higher substrate concentration range (above 10 µl olive oil added per assay), the activity gradually increased with increasing substrate concentrations. We have not yet determined the basis for this pattern, which is unusual with respect to conventional enzyme behavior. Perhaps the rate in this region of substrate concentration was limited by some feature of the assay

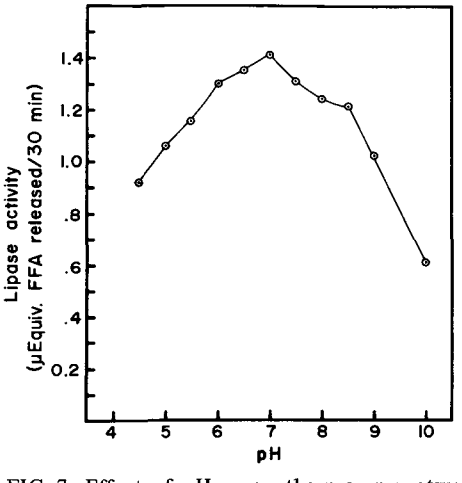

FIG. 7. Effect of pH on southern corn rootworm egg lipase. Enzyme source was extract of an acetone powder of rootworm eggs. See Materials and Methods for other assay details.

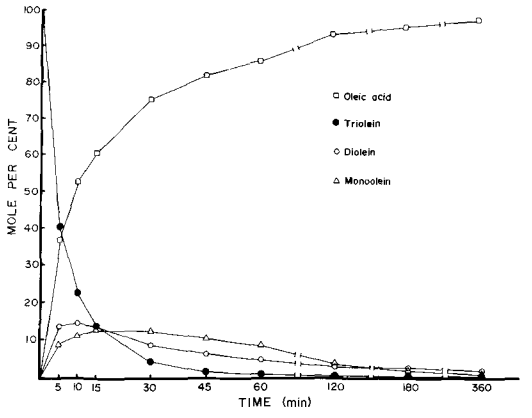

FIG. 8. Changes in relative concentrations of labeled glycerides and free oleic acid during total lipolysis of labeled triolein. Reaction was initiated by adding 0.6 ml of extract of acetone powder of southern corn rootworm eggs (0.186 mg protein/0.6 ml) to following mixture: 1.02 ml radioactively labeled triolein emulsion (see Materials and Methods), 2 ml 0.2 M tris-maleate at pH 7, 0.6 ml 0.075 M $CaCl_2$, 3 mg bovine serum albumin and 3.78 ml H_2O. Aliquots (0.7 ml) were taken at indicated intervals, reaction stopped by addition of 0.1 ml 1 N HCl and distribution of radioactivity among glyceride and oleic acid fractions determined.

method that was counteracted by very high concentrations of substrate.

The effect of temperature on enzyme activity is illustrated in Figure 6. These data are related linearly when plotted according to the Arrhenius equation.

The effect of pH on enzyme activity is summarized in Figure 7. This selected curve is representative of seven series done at several different concentrations of substrate.

To obtain information on the positional capability of the lipase activity of the eggs, we determined the pattern of appearance and disappearance of partial glycerides and oleic acid during the course of total lipolysis of radioactively labeled triolein. The results, summarized in Figure 8, show that the ultimate products of triglyceride (TG) hydrolysis are FFA and glycerol. The same pattern and the same ultimate products were seen in preliminary observations (unpublished) with the two separated isozymes of rootworm egg lipase previously described (10). The possibility that partial glyceride lipase(s) contributed to the total hydrolysis of triolein cannot be ruled out by our observations, but it would require the coincidental migration of such enzymes with the two electrophoretically separable triglyceride lipase isozymes. Several studies (reviewed in References 2 and 18) on lipids and lipases of larval, pupal and adult stages of insects suggest

TABLE I

Activity of Southern Corn Rootworm Egg Lipase towards Triglycerides of Varying Chain Length

	Lipase activity (μeq FFA[a] released/mg protein/min)			
	pH 7		pH 9	
Substrate	Mean	SD	Mean	SD
Triacetin	0		---	
Tripropionin	---		4.90	0.48
Tributyrin	6.37	0.50	5.90	0.23
Trihexanoin	—		6.17	0.13
Trioctanoin	8.74	0.48	10.03	0.47
Tridecanoin	15.48	0.95	14.74	0.24
Triolein	---		11.57	0.31

[a]Free fatty acid.

that partial glycerides are important as mobile forms of fat energy in insects, and thus action of lipases of different positional specificity, acting at different sites, is required for total lipolysis. Indeed the insect lipases thus far studied in some depth are specific for partial glycerides (19-21). Our observations suggest that in the insect egg total lipolysis is the physiologically significant event, although precise studies on highly purified lipase and on egg glycerides are needed.

The data showing the relationship between enzyme activity and the chain length of the acyl moiety of several TG substrates at two different pH values are summarized in Table I. Obtaining data on chain length-activity relationships was complicated by two facts; (a) The rate of autohydrolysis of triacetin was intolerably high for our system at pH 9; and (b) depending on the composition of the assay medium, long chain fatty acids may not be completely ionized at pH values lower than 9 (22). Thus we measured some substrates at pH 7 to observe the relative activity towards triacetin and then did a more extensive series (triacetin excepted) at pH 9. The pattern of activity as a function of acyl chain length was essentially the same in both systems.

Pancreatic lipase is active towards triacetin only when the substrate is emulsified (23). Since triacetin did not yield an emulsion at our standard substrate concentration, we prepared a reaction mixture with triacetin present at 0.656 M, which is two times greater than the saturation concentration. Even under that condition triacetin was not enzymatically hydrolyzed. Incidentally, a pork pancreatic lipase preparation hydrolyzed emulsified triacetin in our system (unpublished observations).

Interpretation of the relationship between the acyl chain length of the substrate and the

activity of a lipase requires caution, because the effect of the nature of the emulsion on the reaction is not known (7) and it might be expected that different glyceride species will yield different emulsion characteristics. Nevertheless the fact that the preparation was actually inactive towards triacetin and increased in activity with increasing chain length suggests that this enzyme is different than the majority of the lipases thus far studied, since in most cases the activity observed decreases with increasing chain length (8).

We have shown that rootworm egg lipase, isolated from nonspecific esterases on the analytical scale by polyacrylamide gel electrophoresis, is active towards naphthyl acetate (10). Yet the enzyme cannot hydrolyze triacetin. We therefore conclude that acyl chain length alone does not determine whether a substrate is acted upon by the lipase. Perhaps, in addition to the presumed interaction of the enzyme with an oil-water interface, the enzyme has in its active site a hydrophobic zone specific for the interaction with lipid substrates. The lipid requirement on the part of the substrate could be fulfilled in TG by long acyl chains and in naphthyl acetate by the naphthol moiety. We are currently attempting to purify SCR egg lipase by preparative means and intend to evaluate the substrate specificity patterns more extensively.

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REFERENCES

1. Fast, P.G., Insect Lipids: a Review, in "Memoirs Entomol. Soc. Canada," No. 37, 1964, 50 p.
2. Gilbert, L.I., Adv. Insect Physiol. 4:69 (1967).
3. Fodor, P.J., Enzymologia 12:343 (1949).
4. Gaeta, I., and A. Zappanico, Ric. Sci. 29:788 (1959).
5. Carlson, L.O., Biol. Bull. 81:375 (1941).
6. Urbani, E., in "Advances in Morphogenesis," Vol. 2, Edited by M. Abercrombie and J. Brachet, Academic Press, New York, 1962, p. 61.
7. Jensen, R.G., in "Progress in the Chemistry of Fats and Other Lipids," Vol. 11, Part 3, Edited by R.T. Holman, 1971, p. 347.
8. Wills, E.D., Adv. Lipid Res. 3:197 (1965).
9. Howe, W.L., and B.W. George, in "Insect Colonization and Mass Production," Edited by C.N. Smith, Academic Press, New York, 1966, p. 367.
10. Guss, P.L., and J.L. Krysan, J. Insect Physiol. 18:1181 (1972).
11. Jensen, R.G., T.A. Marks, J. Sampugna, J.C. Quinn and D.L. Carpenter, Lipids 1:451 (1966).
12. Guss, P.L., Ann. Entomol. Soc. Amer. 62:675 (1969).
13. Krysan, J.L., and P.L. Guss, Biochim. Biophys. Acta 239:349 (1971).
14. Krysan, J.L., and L.E. Chadwick, Entomol. Exp. Appl. 5:179 (1962).
15. Mahadevan, S., C.J. Dillard and A.L. Tappel, Analyt. Biochem. 27:387 (1969).
16. Krysan, J.L., and P.L. Guss, Biochim. Biophys. Acta 296:466 (1973).
17. Brockerhoff, H.J., Biol. Chem. 246:5828 (1971).
18. Chang, F., and S. Friedman, Insect Biochem. 1:63 (1971).
19. Gilbert, L.I., H. Chino and K.A. Domroese, J. Insect Physiol. 11:1057 (1965).
20. Stevenson, E., Ibid. 15:1537 (1969).
21. Stevenson, E., Ibid. 18:1751 (1972).
22. Benzonana, G., and P. Desneulle, Biochim. Biophys. Acta 164:47 (1968).
23. Sarda, L., and P. Desneulle, Ibid. 30:513 (1958).

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Fatty Acid Composition of Phospholipids in Different Regions of Developing Human Fetal Brain

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ABSTRACT

The fatty acid composition of total phospholipids in different regions, viz., cerebrum, cerebellum and medulla oblongata, of developing human fetal brain was studied. All the brains analyzed in the present investigations were obtained from fetuses whose mothers belonged to the poor socioeconomic section of the population. Palmitic, oleic and stearic acids were found to be the dominant fatty acids, and the pattern was similar in all regions of the brain studied between the ages of 22 and 35 weeks. However at birth there appeared to be an increase in polyenoic acids at the expense of lower chain fatty acids, and these changes were of relatively higher magnitude in the cerebellum than in other regions studied. These changes, in terms of increase in polyunsaturated fatty acids near full term, coincided well with the already observed timing of an overall growth spurt of the brain a few weeks preceding birth.

In order to elucidate the possible effects of maternal malnutrition of fetal brain development, studies on the chemical composition of human fetal brain at different stages of intrauterine growth have been undertaken in these laboratories (1,2).

In studies concerned with the growth and maturation of an organ, the qualitative changes in various chemical constituents, in addition to quantitative changes, should be taken into account. This aspect becomes exceedingly important in the case of lipids, as it is now well recognized that the fatty acid composition of a given class or species of a lipid component could have great influence on the chemical and therefore the functional properties of that lipid molecule. In this investigation, the fatty acid composition of different classes of lipids in developing human fetal brain has been studied. In this paper, detailed results of the study on fatty acid profiles in the phospholipid fraction are presented. A brief mention of some of these results has been made in a preliminary report (3).

EXPERIMENTAL PROCEDURES

Fetal brains of different gestational ages ranging from 11 weeks to full term were obtained from a local hospital. The brains were mostly obtained from legal abortions for socio-

TABLE I

Fatty Acid Composition of Total Phospholipids in Cerebrum of Developing Fetal Brain

Gestational age, weeks	Percentage of total methyl esters											
	Fatty acid carbon no. and unsaturation											
	14	16	16:1	18	18:1	18:2	18:3	20:4	22:4 (ω6)	22:5 (ω3)	22:6 (ω3)	Others[a]
11-13 (3)[b]	2.9[c]	36.7	5.2	23.7	26.1	0.2	0.1	3.4	1.1	0.2	0.1	---
20-22 (4)	3.3	30.9	5.3	22.5	19.6	1.1	0.2	7.9	3.1	2.7	2.2	1.6
24-26 (3)	3.9	35.6	7.4	20.9	21.3	0.2	0.2	6.3	3.1	1.9	1.0	---
30-32 (4)	3.5	35.1	5.2	21.7	23.0	0.4	0.8	6.1	2.7	1.2	1.3	0.7
33-35 (4)	3.1	37.1	5.1	24.7	18.4	0.1	0.6	6.2	2.8	1.8	1.1	1.3
Term (4)	1.4	24.1	4.3	26.7	20.3	1.4	1.1	7.3	4.5	2.8	3.4	2.5

[a]Include other unsaturated fatty acids with retention time higher than 20 and 22 carbon units.
[b]Figures in parentheses indicate number of brains analyzed.
[c]Mean values for number of brains analyzed.

TABLE II

Fatty Acid Composition of Phospholipids in Cerebellum of Developing Fetal Brain

Gestational age, weeks	Percentage of total methyl esters: Fatty acid carbon no. and unsaturation											
	14	16	16:1	18	18:1	18:2	18:3	20:4	22:4 (ω6)	22:5 (ω3)	22:6 (ω3)	Others[a]
20-22 (3)[b]	2.0[c]	20.9	3.3	29.7	15.8	2.4	1.0	9.7	5.9	3.0	2.9	3.0
24-26 (3)	2.5	26.0	8.5	21.8	24.6	1.3	1.2	5.3	3.3	0.8	0.9	1.8
30-32 (3)	2.2	27.6	10.3	22.4	22.4	1.3	0.6	5.3	3.4	1.4	1.3	1.4
34 (1)	2.2	32.4	11.1	26.2	19.6	0.2	0.1	5.9	1.6	0.6	1.4	0.9
Term (4)	1.5	22.8	4.7	24.6	18.3	1.3	1.5	8.0	6.0	5.0	4.7	2.2

a,b,cSee footnotes to Table I.

medical reasons and from stillbirths. (All fetuses supplied for the present study belong to the poor socioeconomic group of Indian population, subsisting generally on diets providing ca. 1800 calories and 40 g vegetable protein daily.) In no case was the time between death and procurement of the fetus more than 4 hr. Usually the brain was dissected immediately; if dissection could not be carried out immediately the fetuses were stored at -20 C. Whenever possible, the brains were separated into the anatomically distinct parts, i.e., cerebrum, cerebellum and medulla oblongata. All brains samples were frozen and stored at -20 C prior to lipid extraction.

ISOLATION OF PHOSPHOLIPIDS

A 20% homogenate in glass-distilled water was prepared for all brain samples. A known aliquot of the homogenate was used for extraction of total lipids using chloroform-methanol 2:1 according to Folch et al. (4). The total lipids were dissolved in a minimal amount of chloroform and placed on a silicic acid column. Neutral lipids, glycolipids and phospholipids were eluted successively according to the procedure described by Vorbeck and Marinetti (5). Further purification of the phospholipid fraction was carried out with thin layer chromatography using the solvent system, chloroform-methanol 100:20. Phospholipids were detected by exposure to iodine vapors. Various standard phospholipids were run simultaneously. Under these conditions the phospholipids showed very little mobility, the highest being that of phosphatidylethanolamine (Rf 0.3), whereas all the

TABLE III

Fatty Acid Composition of Total Phospholipids in Medulla Oblongata of Developing Fetal Brain

Gestational age, weeks	Percentage of total methyl esters: Fatty acid carbon no. and unsaturation											
	14	16	16:1	18	18:1	18:2	18:3	20:4	22:4 (ω6)	22:5 (ω3)	22:6 (ω3)	Others[a]
20-22 (2)[b]	3.6[c]	33.0	8.4	21.3	23.4	0.1	0.1	3.1	2.3	1.7	2.3	—
24-26 (3)	2.7	27.6	7.7	22.6	21.8	1.76	0.6	6.2	3.7	0.8	1.1	3.3
30-32 (3)	1.9	27.0	5.2	21.6	26.2	1.6	0.6	7.2	3.9	1.7	2.5	0.2
33-35 (3)	1.9	38.1	4.2	23.1	25.5	0.1	1.1	3.2	1.4	2.2	1.0	—
Term (3)	1.5	20.0	3.4	26.6	22.2	1.3	1.4	6.3	5.5	3.2	2.7	4.2

a,b,cSee footnotes to Table I.

TABLE IV

Distribution of Fatty Acid Groups in Different Regions of Developing Fetal Brain

Gestational age, weeks	Percentage of total methyl esters[a]											
	Nature of fatty acid group											
	Saturated			Unsaturated			Monoenes			Polyenes[b]		
11-13 Whole brain	63.3			36.7			31.3			4.7		
20-22	CB	CL	MO	CB	CL	MO	CB	CL	MO	CB	CL	MO
20-22	56.7	52.8	58.0	43.3	47.2	42.1	24.9	18.1	32.3	16.3	21.1	9.4
24-26	56.5	50.3	49.7	43.5	49.7	47.1	28.7	33.1	29.5	12.4	11.1	14.1
30-32	60.5	52.2	50.5	39.5	47.8	49.5	23.2	32.7	31.4	11.6	12.0	15.1
33-35	64.9	60.8	63.1	35.1	39.2	36.9	22.2	30.7	29.7	12.8	9.8	8.8
Term	52.2	48.9	48.0	47.8	51.1	52.8	24.6	23.0	25.6	18.9	26.2	18.2

[a]CB = cerebrum, CL = cerebellum, and MO = medulla oblongata.
[b]Include tetra-, penta- and hexaenoic acids of 20 and 22 carbon units only.

other contaminating lipids (neutral lipids and glycolipids, etc.) separated with higher Rf.

Preparation of methyl esters of fatty acids and their analysis: Purified phospholipid samples (<10 mg) were dissolved in 0.2 ml benzene in stoppered glass tubes. Five milliliter 5% (w/v) methanolic hydrochloric acid was added. The tightly stoppered tubes, after flushing with N_2 gas, were kept at 80 C for 4 hr. The methyl esters were extracted three times with 5 ml portions of petroleum ether after addition of 10 ml H_2O. The procedure followed here is the combination of the methods described by Stoffel et al. (6) and by Stallberg-Stenhagen and Svennerholm (7). The pooled petroleum ether extracts were rinsed with 5 ml 5% (w/v) solution of sodium bicarbonate and twice with 5 ml portions of distilled water. The extracts were dried over anhydrous sodium sulphate. The residue, after evaporation in vacuo and finally under N_2, was dissolved in a small amount of chloroform and stored under N_2 in tightly stoppered glass tubes prior to gas liquid chromatography.

The methyl esters of total phospholipids were analyzed by gas liquid chromatography using a Wilkens Model 650 Aerograph equipped with hydrogen flame ionization detector. A 6 ft copper column (1/8 ID) packed with 15% diethylene glycol succinate coated on Chromosorb W (80-100 mesh silanized) was used. The carrier gas was nitrogen with a flow rate of 30 ml/min. The oven temperature was 200 C.

Standard fatty acid methyl esters supplied by (Sigma Chemical Co.) were used for identification. Fatty acids for which standards were unavailable were tentatively identified by carbon number, by plotting log retention time against chain length or degree of unsaturation (8) and by rechromatography of the samples after hydrogenation.

RESULTS AND DISCUSSION

The fatty acid profiles of phospholipids of developing human brain regions are shown in Tables I-III.

In general, palmitic, stearic and oleic acids were the dominant fatty acids irrespective of the gestational age and the region of the brain. These three fatty acids together constituted ca. 86% of the total fatty acids in whole brains of gestational ages between 11 and 13 weeks. Their concentration varied between 70 and 80% in different regions during the period between the 22nd and 35th weeks of intrauterine life. These values, however, diminished slightly around full term (cerebrum 71%, cerebellum 65.7% and medulla oblongata 68.8%).

Myristic (14:0) and palmitoleic (16:1) acids along with palmitic acid (16:0) showed a reduction in concentration at term as compared to earlier gestational periods, i.e., values up to 35 weeks. These changes were similar in all three regions of the brain, except that palmitic and myristic acid levels were of relatively higher magnitude in the cases of cerebrum and medulla oblongata as compared to cerebellum.

Oleic acid (18:1) constituted the major unsaturated fatty acid in all three regions of the brain. The dienoic and trienoic 18, 20 and 22 carbon fatty acids were very low, accounting for less than 1% at earlier gestational ages.

Arachidonic acid (20:4) was the second most prevalent unsaturated fatty acid throughout the intrauterine development of the brain. The levels of this fatty acid varied 6-8% in the cerebrum, 5-9% in the cerebellum and 3-7% in the medulla oblongata. Polyunsaturated fatty acids of 22 carbon chain lengths constituted

5.2-10.7% in the cerebrum, 3.6-15.7% in the cerebellum and 5.6-11.4% in the medulla oblongata. Of these, two fatty acids were tentatively identified as 22:4 (ω6) and 22:5 (ω3), as the retention times of their peaks corresponded well with those reported by O'Brien and Sampson (9). The fatty acid designated as docosapentaenoic acid of the linoleic acid series (22:5ω6) by O'Brien and Sampson, was later designated as 22:4 (ω6) by Svennerholm (10) based on mass spectrometric analysis, since the work of Svennerholm was more recent, we have retained the latter designation, i.e., 22:4ω6. The concentration of this fatty acid was generally more than other 22 polyenoic acids at all gestational ages, irrespective of the brain region studied.

Table IV gives the relative percentage of fatty acids when grouped as saturated, monoenoic and polyenoic acids. The concentration of polyenes at earlier gestation of 11-13 weeks (for the whole brain) was very low. However no clear cut differences were noticed between the regions with regard to saturates, monoenoates and polyenoates between 22 and 35 weeks of gestation. But, at term, the levels of saturated fatty acids showed a reduction that was compensated by an increase in polyenoic acids. A greater concentration of total polyenes was noted in the cerebellum at term, although in this region the levels of polyunsaturates, even at 22 weeks of gestation, were fairly high. The level of polyenoic acids in the cerebellum was 26.2% at term, compared to 18.9 and 18.2% for cerebrum and medulla oblongata, respectively.

These data may perhaps be considered indicative of a rise in the activities of those brain enzymes responsible for chain elongation and desaturation of fatty acids between the 35th week of gestation and birth. It is also possible that the higher levels of polyunsaturates were actually due to increased transport of these fatty acids. It is significant that during this period of intrauterine life a marked increase in several chemical constituents, viz., DNA, protein, lipid, was also observed (1,2,11). It therefore appears that, a few weeks prior to birth, not only does the total lipid content of human fetal brain show a sudden rise, but at the same time qualitative changes also take place, as shown by the present results in the increased levels of polyenoic acids.

O'Brien and Sampson (9), Svennerholm (10) and Altrock and Debuch (12) have reported the fatty acid composition of phospholipids of human brains of different ages. The present data cannot be strictly compared with those results, since whole brain (12), or the gray and white matter separately, and different species of phospholipids were used in these analyses (9,10). It is, however, pertinent that the fatty acids of phospholipids in different regions of the brain reported in this study are similar to those reported by Altrock and Debuch (12) for whole brain. These latter workers also observed a decreasing trend in the levels of myristic, palmitic and palmitoleic acids with advancing gestation.

The extensive studies of Svennerholm on the changes in fatty acid composition of phospholipids in human brains of varying ages indicated that in younger brains the linoleate series (from ethanolamine phosphoglyceride) predominate over the linolenate series and that the former diminish with corresponding increase in the latter, with the maturity of the brain. It is interesting that, within the period studied, the present results also show a similar trend, i.e., an increase in the ratio of the linolenic acid series to the linoleic acid series at term as compared to earlier periods of gestation.

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REFERENCES

1. Subba Rao, K., and M.K. Janardana Sarma, Proc. Nutr. Soc. India 12:8 (1972).
2. Subba Rao, K., M.K. Janardana Sarma and P. Srinivasa Rao, "Proceedings of the ICMR Young Scientific Research Workers' Conference, 1972, Hyderabad," Indian Council of Medical Research, Technical Bulletin Series 16, In press.
3. Srinivasa Rao, P., and K. Subba Rao., Biochem. J. 128:44P (1972).
4. Folch, J., M. Lees. and G.H. Sloane-Stanley, J. Biol. Chem. 226:497 (1957).
5. Vorbeck, M.L., and G.V. Marinetti, Biochemistry 4:296 (1965).
6. Stoffel, W., C. Florence and H.A. Edward. Analyt. Chem. 31:307 (1959).
7. Stallberg-Stenhagen, S., and L. Svennerholm, J. Lipid Res. 6:146 (1965).
8. Woodford, F.P., and C.M. van Gent, Ibid. 1:188 (1960).
9. O'Brien, J.S., and E.L. Sampson, Ibid. 6:545 (1965).
10. Svennerholm, L., Ibid. 9:570 (1968).
11. Winick, M., Pediat. Res. 1:352 (1968).
12. Altrock, K., and H. Debuch, J. Neurochem. 15:1351 (1968).

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Differential Effects of Benzodioxane, Chroman and Dihydrobenzofuran Analogs of Clofibrate in a Triton Hyperlipemic Rat Model

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ABSTRACT

Clofibrate (ethyl 2-methyl-2-[4-chlorophenoxy] propionate) is currently an important hypolipemic agent. In this study we describe the biological properties of certain acyclic and cyclic analogs of clofibrate in a hyperlipemic rat model in which the hyperlipemia was induced by ip injection of Triton WR-1339. Cyclic analogs studied for their hypocholesterolemic and hypotriglyceridemic activities, as well as their ability to modify lipoprotein patterns, include the ethyl esters of 1,4-benzodioxane-2-carboxylic acid, 6-chlorochroman-2-carboxylic acid and 5-chloro-2,3-dihydrobenzofuran-2-carboxylic acid. Among the clofibrate analogs, ethyl 6-chlorochroman-2-carboxylate compares most favorably with the parent compound. Whereas the 6-chlorochroman-2-carboxylate is effective as a hypocholesterolemic and hypotriglyceridemic agent, the 1,4-benzodioxane analog exhibits mainly hypotriglyceridemic activity, while the 2,3-dihydrobenzofuran analog exhibits hypocholesterolemic activity. Except for the benzodioxane, deschloro analogs are inactive. Results obtained in these studies are discussed in terms of structural requirements for biological activity and modes of action proposed for the parent drug clofibrate.

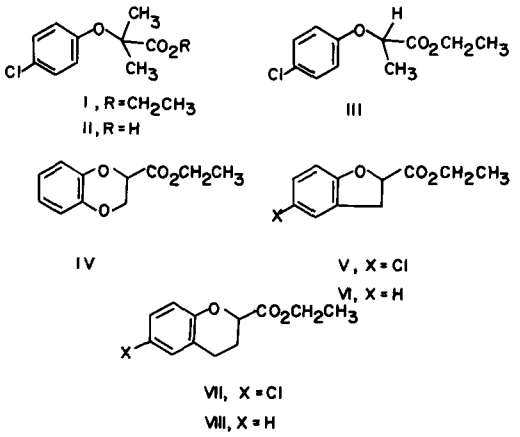

FIG. 1. Structures for analogs of clofibrate (I); I = ethyl 2-methyl-2-(4-chlorophenoxy)propionate; II = 2-methyl-2-(4-chlorophenoxy)propionic acid; III = ethyl D,L-2-(4-chlorophenoxy)propionate; IV = ethyl 1,4-benzodioxane-2-carboxylate; V = ethyl 5-chloro-2,3-dihydrobenzofuran-2-carboxylate; VI = ethyl 2,3-dihydrobenzofuran-2-carboxylate; VII = ethyl 6-chlorochroman-2-carboxylate; VIII = ethyl chroman-2-carboxylate.

INTRODUCTION

For purposes of defining minimum structural requirements for maximum antilipemic action of compounds related to clofibrate (ethyl 2-methyl-2-[4-chlorophenoxy] propionate [I]), probing the nature of biochemical sites that are either blocked or stimulated by this hypolipemic drug, classifying such sites according to their selective affinity or intrinsic activity towards analogs resulting from minor molecular modification of I, providing insight into the mechanism(s) of action of such analogs and developing new leads for the design of hypolipemic drugs that have potential use in the treatment of atherosclerosis (1), Witiak and coworkers (2-5) synthesized for such biological investigations (2-9) a series of acyclic and cyclic analogs III-VIII. Preliminary investigations in vitro employing the hydrolysis product of I, namely II, which is presumed to be the active hypolipemic agent in vivo due to rapid metabolism by tissue and serum esterases (10), showed II to have properties similar to those observed for the free acid hydrolysis products of ethyl D,L-2-(4-chlorophenoxy)propionate (III), a simple desmethyl analog of I; ethyl 1,4-benzodioxane-2-carboxylate (IV), an analog derived by bonding the α-methyl group of deschloro III via an ether linkage to the ortho position of the phenyl ring; ethyl 5-chloro-2,3-dihydro-2-benzofurancarboxylate (V) and 2,3-dihydro-2-benzofurancarboxylate (VI), analogs derived by bonding the α-methyl group of III or deschloro-III, respectively, directly to the ortho position of the phenyl ring; and ethyl 6-chlorochroman-2-carboxylate (VII) and ethyl chroman-2-car-

boxylate (VIII), derived by bonding the α-methyl group of III or deschloro-III, respectively, via a methylene group to the ortho position of the phenyl ring (Fig. 1).

For these reasons we anticipated that analogs III-VIII would exhibit qualitatively similar hypolipemic activity in vivo and that quantitative differences in potency observed could be interpreted in light of mechanism studies obtained in vitro or in vivo for these analogs. In the present study we report the results of experiments designed to assess the hypolipemic efficacy of analogs III-VIII in hyperlipemic rats, in which the hyperlipemia was induced by ip injection of Triton WR-1339 (11). The hypocholesterolemic and hypotriglyceridemic activities of these drugs, as well as their ability to modify lipoprotein patterns, are compared with results obtained for clofibrate.

METHODS

Analogs I-VIII were synthesized according to methods previously reported by Witiak and coworkers (2-5). These compounds were tested in a hyperlipemic rat model (11) in which the hyperlipemia was induced by ip injection of Triton WR-1339 (oxyethylated tertiaryoctylphenolformaldehyde polymer, Ruger Chemical Co., Philadelphia, Pa.). Male albino rats (Sprague-Dawley) were housed in groups of six and were fed Purina laboratory chow and water ad libitum for a 2 week stabilizing period. After this period the rats were redistributed by weight into four experimental groups of six rats each. Two experimental groups, weighing 260-280 g per rat, were fasted for 24 hr and then injected ip with 225 mg Triton per kilogram dissolved in 0.15 M NaCl to give a concentration of 62.5 mg/ml. The two control groups of comparable weight received 2 ml only of the vehicle (0.25% aqueous methyl cellulose), whereas the remaining groups received test compounds in vehicle. Compounds were dispersed in the vehicle at concentrations of 2 mg/ml, providing a total screening dose of 0.30 mg/kg for 270 g rats in 4 ml. Each rat received 2 ml doses by gastric intubation, the first immediately after the Triton injection and the second 20 hr later. Fasting was continued during the post-Triton period.

At 43 hr after Triton administration the rats were anesthetized with ethyl ether; blood was drawn from the abdominal aorta and added to ethylenediaminetetraacetic acid (EDTA, 0.9 mg/ml); and plasma was obtained after centrifugation at 500 x g for 10 min. Plasma triglyceride was determined by the method of Eggstein (12); plasma cholesterol was analyzed by the method of Parekh and Jung (13). Lipoproteins were separated electrophoretically by the method of Noble (14) with Pfizer prepared agarose gels. The staining was carried out for 4 min with micellar Fat Red 7B. The agarose gels were washed in chloroform-methanol 2:1 v/v for 20 sec. The percentages of Fat Red 7B dye uptake into lipoproteins were determined by densitometric scanning and integration of the lipoprotein pattern. Significant differences in plasma cholesterol and triglyceride concentrations between drug-treated and control groups were determined by student t tests on logarithms of individual data to allow the pooling of variances. To test for significance among lipoprotein patterns a student t test was performed on the arc sine $\sqrt{x_i}$ in which x_i represents the original percentage of dye uptake.

RESULTS

The effects of clofibrate (I) and its analogs on plasma cholesterol concentrations were tested in control male Sprague-Dawley Purina chow-fed and Triton-induced hyperlipemic rats. In Triton-hyperlipemic animals (Table I) clofibrate (I) and all the analogs, with the exception of two deschloro compounds VI and VIII and desmethyl analog III, significantly reduced plasma cholesterol levels by 30-74% of those of hyperlipemic controls (group III). The one poorly controlled factor in these observations was the variability in the Triton hypercholesterolemia. In order to define and evaluate the effectiveness of these drugs in lowering cholesterol levels in hyperlipemic animals, groups I and IV were tested for significant differences. When analyzed in this manner, only the cyclic chloro analogs V and VII compared favorably with clofibrate (I), i.e., there was no statistically significant difference between the plasma cholesterol concentrations in drug-treated Triton hyperlipemic and control rats. While the desmethyl analog III lowered cholesterol levels significantly in normocholesterolemic rats (compare groups I and II), the apparent lowering of serum cholesterol levels in hyperlipemic rats was not significant. The only other analog having hypocholesterolemic activity in normocholesterolemic Sprague-Dawley rats was ethyl 5-chloro-2,3-dihydrobenzofuran-2-carboxylate (V).

The effects of clofibrate (I) and its analogs on plasma triglyceride concentrations also were examined in Purina chow-fed and Triton-induced hyperlipemic rats (Table II). In Triton-induced hyperlipemic rats significant reductions in the plasma triglyceride concentrations (ranging from 32 to 96% of hyperlipemic controls; group III) were demonstrated with clofibrate

TABLE I

Effect of Clofibrate and Its Analogs on Plasma Cholesterol Levels in Male Sprague-Dawley Rats

Compound	Control group (I)	Drug-treated control (II)	Triton hyperlipemic (III)	Drug-treated Triton hyperlipemic (IV)
Clofibrate (I)	60.6 ± 7.2 (5)[a]	62.4 ± 8.5	267 ± 85	59.3 ± 12[b]
Ethyl D,L-2-(4-chlorophenoxy)propionate (III)	72.7 ± 12	56.5 ± 11.4[d]	152 ± 37	120 ± 58
Ethyl 1,4-benzodioxane-2-carboxylate (IV)	83.1 ± 18	74.0 ± 21.0	347 ± 70	124 ± 42[b,d]
Ethyl 5-chloro-2,3-dihydrobenzofuran-2-carboxylate (V)	97.8 ± 10.8	55.0 ± 11.7[c]	177 ± 63	93.0 ± 19[b]
Ethyl 2,3-dihydrobenzofuran-2-carboxylate (VI)	54.2 ± 7.5	59.2 ± 6.7	142 ± 25	142 ± 32[d]
Ethyl 6-chlorochroman-2-carboxylate (VII)	57.6 ± 5.0	52.0 ± 8.4	249 ± 116	64.0 ± 22[b]
Ethyl chroman-2-carboxylate (VIII)	69.0 ± 17	71.0 ± 19.9	354 ± 45	325 ± 66[d]

[a]Mean ± SD; six rats unless otherwise noted by number in parentheses.

[b]Statistically significant $P < 0.05$; Triton hyperlipemic vs. drug-treated Triton hyperlipemic (comparison of group III and IV).

[c]Statistically significant $P < 0.05$; drug-treated control vs. control (comparison of groups I and IV).

[d]Statistically significant $P < 0.05$; drug-treated Triton-hyperlipemic vs. control (comparisons of groups I and IV).

and all analogs, excluding dihydrobenzofurans V and VI and the desmethyl analog III. One potential perturbation in these measurements is that in hyperlipemic controls, plasma triglyceride varied from 92.9 to 1324 mg%. There was no significant plasma triglyceride lowering by either clofibrate (I) or its derivatives in nonhyperlipidemic Purina chow-fed Sprague-Dawley rats, i.e., no significant lowering was observed in plasma triglyceride concentrations in group II when compared to group I.

In contrast to results obtained for hypocholesterolemic activity, when the efficacy of these compounds was assessed in terms of significant differences of the mean serum triglyceride level for groups I and IV, only clofibrate (I) and ethyl 1,4-benzodioxane-2-carboxylate (IV) showed no significant difference. None of the other experimental compounds were this effective as hypotriglyceridemic agents. The next

TABLE II

Effect of Clofibrate and Its Analogs on Plasma Triglyceride Levels in Male Sprague-Dawley Rats

Compound	Control group (I)	Drug-treated control (II)	Triton hyperlipemic (III)	Drug-treated hyperlipemic (IV)
Clofibrate (I)	20.9 ± 6.2 (5)[a]	30.5 ± 8.2	774 ± 329	27.6 ± 15[b]
Ethyl D,L-2-(4-chlorophenoxy)propionate (III)	11.2 ± 4.9	51.4 ± 32.3	92.8 ± 34.6	64.0 ± 45[c] (5)
Ethyl 1,4-benzodioxane-2-carboxylate (IV)	28.8 ± 13	35.4 ± 3.4	443 ± 292	51.3 ± 25[b]
Ethyl 5-chloro-2,3-dihydrobenzofuran-2-carboxylate (V)	56.4 ± 29	34.3 ± 8.7	162 ± 121	109 ± 59[c]
Ethyl 2,3-dihydrobenzofuran-2-carboxylate (VI)	31.2 ± 19	22.1 ± 6.6	127 ± 50	128 ± 88[c]
Ethyl 6-chlorochroman-2-carboxylate (VII)	12.5 ± 6.3	22.8 ± 4.7	526 ± 226 (5)	40.7 ± 24[b,c]
Ethyl chroman-2-carboxylate (VIII)	34.0 ± 18	25.1 ± 13.6	1,324 ± 207	659 ± 193[b,c]

[a]Mean ± SD; six rats unless otherwise noted by number in parentheses.

[b]Statistically significant $P < 0.05$; Triton hyperlipemic vs. drug-treated Triton hyperlipemic (comparison of groups III and IV).

[c]Statistically significant $P < 0.05$; drug-treated hyperlipemic vs. control (comparison of groups I and IV).

most effective hypotriglyceridemic agent was the 6-chlorochroman analog VII, which, unlike benzodioxane IV, also has good hypocholesterolemic activity.

The reduction in plasma lipid levels in hyperlipemic rats should be reflected in a change in the distribution of lipoproteins in rat plasma. This change is demonstrated by examining representative electrophoretically derived lipoprotein patterns from plasma of hyperlipemic and control rats treated with clofibrate. Three distinct bands are discernible in the experimental and control groups (Fig. 2). With Triton-induced hyperlipemia band A is significantly increased, whereas the relative percentage of bands B and C are concomitantly reduced. Clofibrate treatment returns these lipoprotein patterns to the distributions found in controls.

The effect of clofibrate and its analogs on Fat Red 7B dye uptake into electrophoretically separated lipoproteins is summarized in Table III. Each experimental group is composed of six rats unless otherwise specified in the table. Significant increases in band A, with concomitant decreases in bands B and C, were observed in all hyperlipidemic rat plasmas after Triton administration. Thus the efficacy of hyperlipemia-reducing compounds also may be assessed by determining whether the effect of the drug is sufficient to render the shift in plasma lipoprotein pattern back to normal. A particularly effective drug would be a compound that not only returns the serum cholesterol and triglyceride levels to normal, but also shows no significant difference between groups I and IV when bands A, B and C are measured by densitometric scan of lipoprotein fractions after Fat Red 7B dye uptake.

Significant shifts of plasma lipoprotein distribution back to the control pattern were particularly pronounced in clofibrate-treated animals. Whereas dihydrobenzofuran V administration did affect a distribution of lipoprotein back to normal in terms of relative per cent of bands A, B and C, this compound is quantitatively less effective than I as a hypotriglyceridemic agent. All chlorinated analogs, more so than deschloro analogs, exhibited the ability to return the lipoprotein distribution patterns toward normal, but none were as efficacious as clofibrate.

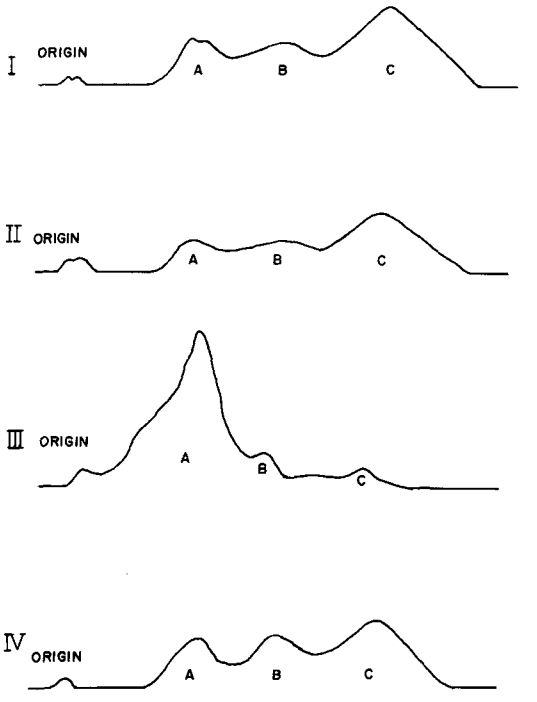

FIG. 2. Representative densitometric scans of Fat Red 7B dye uptake into lipoprotein fractions (bands A, B and C) in rat serum. Panel I = control rats; panel II = clofibrate-treated control; panel III = Triton-hyperlipemic rats; panel IV = clofibrate-treated Triton-induced hyperlipemic rats.

DISCUSSION

In an overall comparison of the hypolipemic activity of these experimental compounds with clofibrate (I), it would appear that the 6-chlorochroman ester VII compares most favorably. However none of the experimental drugs are as effective as clofibrate (I), when assessed for their hypocholesterolemic and hypotriglyceridemic activities as well as their ability to return plasma lipoprotein patterns in hyperlipemic rats back to normal. It is also of interest to note that the benzodioxane ester IV, which has no chloro substituent, is an effective hypotriglyceridemic agent that has moderate hypocholesterolemic activity. Based on data discussed in this report, we expect that insertion of chlorine atoms *para* to the ether oxygens in IV will enhance the biological activity in the benzodioxane series; such studies are presently under investigation in our laboratories. Whereas we previously observed deschloro clofibrate to have hypocholesterolemic activity in normocholesterolemic Swiss Webster rats (3), removal of the chloro groups or replacement with other functions generally affords less active or inactive analogs (3,15).

From these studies it is clear that apparently minor molecular modification of the parent drug clofibrate (I) may result in major differences in biological activity. For example, whereas the structurally constrained chloro-substituted dihydrobenzofuran ester V is effective

TABLE III

Densitometric Scan of Fat Red 7B Dye Uptake into Electrophoretically Separated Lipoprotein Fractions from Plasma of Control (I), Drug-Treated Control (II), Triton Hyperlipemic (III) and Drug-Treated Triton Hyperlipemic (IV) Rats

Compound	Experimental group	Per cent of total peak area		
		Band A	Band B	Band C
Clofibrate (I)	I	21 ± 7(5)[a]	16 ± 7(5)	63 ± 4(5)
	II	21 ± 4	13 ± 6	66 ± 9
	III	92 ± 4	2 ± 1	6 ± 3
	IV	26 ± 8[b]	14 ± 4[b]	60 ± 11[b]
Ethyl D,L-2-(4-chlorophenoxy)-propionate (III)	I	20 ± 4	15 ± 2	65 ± 5
	II	21 ± 5	18 ± 8	61 ± 11
	III	79 ± 19	10 ± 9	11 ± 10
	IV	43 ± 20[b,c]	19 ± 7	38 ± 16[b,c]
Ethyl 1,4-benzodioxane-2-carboxylate (IV)	I	26 ± 6	29 ± 5	45 ± 4
	II	22 ± 5	18 ± 5	60 ± 6
	III	96 ± 1	1 ± 0	3 ± 1
	IV	61 ± 26[b,c]	15 ± 9[b,c]	24 ± 17[b,c]
Ethyl 5-chloro-2,3-dihydro-benzofuran-2-carboxylate (V)	I	25 ± 6	21 ± 8	54 ± 8
	II	25 ± 2	15 ± 4	65 ± 10
	III	89 ± 9	8 ± 10	3 ± 4
	IV	27 ± 20[b]	20 ± 8[b]	53 ± 21[b]
Ethyl 2,3-dihydrobenzo-furan-2-carboxylate (VI)	I	22 ± 5	22 ± 3	56 ± 5
	II	22 ± 12	24 ± 6	54 ± 9
	III	96 ± 8	2 ± 1	2 ± 2
	IV	84 ± 6[b,c]	9 ± 6[b,c]	7 ± 3[b,c]
Ethyl 6-chlorochroman-2-carboxylate (VII)	I	20 ± 3	15 ± 2	65 ± 4
	II	18 ± 1	19 ± 2	63 ± 2
	III	95 ± 2	2 ± 1	3 ± 1
	IV	43 ± 13[b,c]	20 ± 4[b,c]	37 ± 11[b,c]
Ethyl chroman-2-carboxylate (VIII)	I	21 ± 7	22 ± 4	57 ± 5
	II	18 ± 5	24 ± 7	58 ± 5
	III	97 ± 1	1 ± 0	2 ± 0
	IV	82 ± 14[b,c]	10 ± 8[b,c]	8 ± 7[b,c]

[a]Mean ± SD; six rats unless otherwise noted by number in parentheses.

[b]Statistically significant $P < 0.05$; Triton hyperlipemic vs. drug-treated Triton hyperlipemic (comparison of groups III and IV).

[c]Statistically significant $P < 0.05$; drug-treated Triton hyperlipemic vs. control (comparison of groups I and IV).

as a hypocholesterolemic drug and returns the relative percentage of lipoproteins back to normal, it is essentially inactive as a hypotriglyceridemic agent. On the other hand, the cyclic benzodioxane ester is more effective as a hypotriglyceridemic agent and has only moderate hypocholesterolemic activity. Insertion of a methylene group into the five-membered ring of dihydrobenzofuran V affords the conformationally less rigid 6-chlorochroman VII, which has both good hypocholesterolemic and hypotriglyceridemic activity. However this analog is only moderately effective as a modifier of hyperlipemic lipoprotein patterns.

In previous studies it was shown that only the L(S)-ester of III had hypocholesterolemic activity in normocholesterolemic Swiss Webster rats (3); the D(R)-enantiomorph was biologically inactive in such rats when this compound was administered for 12 days mixed with the diet in a concentration of 0.5%. Under these conditions the L(S)-isomer was also a somewhat less potent hypocholesterolemic agent than clofibrate (I). In the present studies we also observed low hypocholesterolemic activity with D,L-III in normocholesterolemic rats; no activity was observed in hyperlipemic rats. The rigid dihydrobenzofuran analog (V) of III also exhibited hypocholesterolemic activity in normal Sprague-Dawley rats. Whereas these results might be explained on the basis of different lipoprotein carriers in normal and hypolipemic rats (note band A, Fig. 2 has more than one component in hyperlpemic rats), we prefer not to speculate further at this time since clofibrate is inactive in normal rats under the conditions of our experiment.

It would seem that resolution of asymmetric cyclic analogs IV, V and VII is desirable; optically pure compounds having the appropriate absolute configuration, i.e., S, may exhibit superior hypolipemic activity. Additional studies with structurally modified analogs of IV, V and VII are indicated since clofibrate (I)

is effective mainly in the treatment of patients with hyperlipidemia Types III, IV and V (16,17).

Whereas the Triton model may be predictive of hyperlipoproteinemia Types IV and V, it should be understood that Triton WR-1339 will modify chylomicrons and other lipoproteins, resulting in a reduced enzymatic hydrolysis catalyzed by lipoprotein lipase causing increased serum triglyceride levels, unlike the human situation in which the hyperlipemia does not result from this type of mechanism (18). Elevated cholesterol levels may be due in part to a modification of the low density lipoproteins, so that they do not influence negative feedback inhibition in the biosynthesis of cholesterol (19).

In addition to the potential clinical significance of these studies, results obtained in vivo may be analyzed, in light of results obtained in various studies in vitro, to increase our understanding of the modes of action of clofibrate and related analogs. It is generally accepted that clofibrate (I) undergoes rapid hydrolysis in vivo (and in vitro) to the free acid II, which is presumed to be the active hypolipemic agent (3,6,10). Esters III-VIII also undergo rapid hydrolysis in vitro by rat serum esterases (6,20); all analogs including clofibrate are nearly completely hydrolyzed in ca. 5 min. Therefore the difference in hypolipemic activities between these esters cannot be attributed to differences in rates of hydrolysis to the active carboxylic acid. The pKa's for these hydrolysis products also are similar. For the carboxylic acids derived from I, III, IV, V and VII, the pKa's are 4.46, 4.35, 4.23, 4.36 and 4.26, respectively (1). The log P (P = octanol-water partition coefficients) for the respective hydrolysis products of I, III, IV, V, VII and VIII, experimentally determined at a pH 2 units below their respective pKa's, are 2.57, 2.31, 1.40, 2.11, 2.40 and 1.90 (20). These values are in agreement with values calculated from the experimentally determined log P for phenoxyacetic acid (21) and may be used to predict distribution of drugs via a random walk process to sites of action (22). While the two most active hypotriglyceridemic and hypocholesterolemic drugs (I and VII) also have the largest log P values for their corresponding acids, the relative activity of other analogs show no apparent relationship to partition coefficients. Because of the similar pKa's and lack of correlation with log P values observed for these compounds, it seems to us that differences in activity observed in vivo are not due to major differences in absorption and distribution, but rather to structural differences that either enhance or decrease the affinity of the molecule for certain enzymes at sites of action or loss. Sites of loss for these drugs remain to be assessed; qualitative differences in metabolism may account, in part, for differences in hypolipemic activity.

Further evidence, suggesting that the corresponding carboxylic acid analogs of these esters are transported in vitro by binding to plasma proteins, is obtained by regression analysis of Scatchard plots of data obtained from ultrafiltration studies (1). The free acids of all analogs, excluding the hydrolysis product of deschloro-2,3-dihydrobenzofuran VI, bind to rat serum albumin (fraction V) at site K_1 with an association constant 1.8-5.1 x 10^4 and to site K_2 with an association constant of 1.5 x 10^2. The hydrolysis product of the inactive ester VI binds to only one site (K = 3.4 x 10^3). Unpublished data obtained in our laboratories do show that the free acids of analogs I-VIII are bound to the thyroxine-binding site on rat serum albumin. While these data will be discussed in some length in a future publication, we wish to point out at this time that our findings seem rather significant in the light of one interesting theory proposed concerning the mode of action of clofibrate (3,6,23) invoking displacement of endogenous L-thyroxine, which is surmised to be the active hypolipemic agent, from its preferential binding site on rat albumin (24). In other species, thyroxine is preferentially bound to other plasma proteins. Since no parallelism exists between protein-binding parameters and hypolipemic activity, it seems unwarranted to us to use such analyses in vitro in an attempt to predict hypolipemic activity in vivo (23). Drug-binding to serum albumin is probably important for drug transport, but differences in metabolism or affinity for lipid enzyme systems (25,26) probably accounts for quantitative and qualitative differences in hypolipemic and other actions in vivo. These conclusions are in agreement with our earlier observations (6), as well as with those of Chang et al. (24) and Musa et al. (27), who were unable to demonstrate any interference by clofibrate with binding of thyroxine to thyroxine-binding globulin, which is the primary carrier of thyroxine in humans. Recent studies by McKerron et al. (28) and Barbosa and Oliner (29) also fail to reveal any significant effects on free thyroxine levels in man.

Clofibrate administration is associated with depressed incorporation of ^{14}C-pyruvate or ^{14}C-acetate into cholesterol or mevalonate by liver slices, and the observations by White indicate that this drug regulates hepatic cholesterol biosynthesis by inhibiting microsomal reduction of HMG-CoA to mevalonate (25). Inhib-

itory effects in vitro on the incorporation of mevalonate-2-^{14}C into nonsaponifiable material in rat liver homogenate, with the corresponding carboxylic acids of a selected number of our analogs (4,5), are also in agreement with hypocholesterolemic properties observed for the esters in vivo. Esters I, V and VII return serum cholesterol levels in hyperlipemic rats back to normal in vivo; the corresponding carboxylic acids are equally effective inhibitors of cholesterol biosynthesis in vitro. Ester IV is moderately active and ester VIII is inactive as a hypocholesterolemic agent in vivo; their respective carboxylic acids are less potent biosynthetic blockers in vitro. Whereas blocking HMG-CoA reductase in vivo seems to be a major mode of action of clofibrate, our studies in vitro indicate that structural requirements for blocking mevalonic acid incorporation into cholesterol may be similar, i.e., results obtained by us in vitro may be indicative of hypocholesterolemic activity in vivo. On the other hand, there is no apparent relationship between the effect of these esters as hypotriglyceridemic agents in vivo and the ability of the corresponding hydrolysis products to inhibit norepinephrine-induced lipolysis in vitro (4,5). This may be a result of the large variance in triglyceride levels between groups of hyperlipemic rats or an indication that these compounds do not exert their hypotriglyceridemic effect by blocking lipolysis. Further work is necessary before any conclusions can be drawn. The findings of Maragoudakis (26), showing that clofibrate inhibits acetyl-CoA carboxylase, and the findings of Fallon and coworkers (30), showing that clofibrate decreases liver synthesis of *sn*-glycerol-3-P, may in part account for the hypotriglyceridemic activity of this drug. It also has been suggested that clofibrate alters the release of lipoproteins by liver (31). It seems to us that clofibrate probably exerts its hypolipemic effect by multiple modes of action and that apparently small changes in structure cause large changes in affinity for lipid-regulating enzymes.

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REFERENCES

1. Witiak, D.T., D.R. Feller, H.A.I. Newman, T.F. Whayne, N.J. Lewis, W.P. Heilman, T.D. Sokoloski and R. Nazareth, "Abstracts of the 13th National Meeting, APhA Academy of Pharmaceutical Sciences," Vol. 2, No. 2, Chicago, November 5-9, 1972, p. 86.
2. Witiak, D.T., R.E. Hackney and M.W. Whitehouse, J. Med. Chem. 12:697 (1969).
3. Witiak, D.T., T. C.-L. Ho, R.E. Hackney and W.E. Connor, Ibid. 11:1086 (1968).
4. Witiak, D.T., D.R. Feller, E.S. Stratford, R.E. Hackney, R. Nazareth and G. Wagner, Ibid. 14:754 (1971).
5. Witiak, D.T., E.S. Stratford, R. Nazareth, G. Wagner and D.R. Feller, Ibid. 14:758 (1971).
6. Witiak, D.T., and M.W. Whitehouse, Biochem. Pharmacol. 18:971 (1969).
7. Witiak, D.T., T.D. Sokoloski, M.W. Whitehouse and F. Hermann, J. Med. Chem. 12:754 (1969).
8. Chien, Y.W., T.D. Sokoloski, C.L. Olson, D.T. Witiak and R. Nazareth, J. Pharm. Sci. 62:440 (1973).
9. Whayne, T.F., and D.T. Witiak, J. Med. Chem. 16:228 (1973).
10. Thorp, J.M., Lancet 1:1323 (1962).
11. Schurr, P.E., J.R. Schultz and T.M. Parkinson, Lipids 7:68 (1972).
12. Eggstein, M., Klin. Wochenschr. 44:267 (1966).
13. Parekh, A.C., and D.H. Jung, Anal. Chem. 42:1423 (1970).
14. Noble, R.P., J. Lipid Res. 9:693 (1968).
15. Grisar, J.M., R.A. Parker, T. Kariya, T.R. Blohm, R.W. Fleming and V. Petrow, J. Med. Chem. 15:1273 (1972).
16. Lees, R.S., and D.E. Wilson, New Eng. J. Med. 284:186 (1971).
17. Fredrickson, D.S., R.I. Levy and R.S. Lees, Ibid. 276:32, 94, 148, 215, 273 (1967).
18. Schotz, M.C., A. Scanu and I.H. Page, Amer. J. Physiol. 188:399 (1957).
19. Frantz, I.D., Jr., and B.T. Hinkelman, J. Exp. Med. 101:225 (1955).
20. Nazareth, R.I., T.D. Sokoloski, D.T. Witiak and A.T. Hopper, J. Pharm. Sci., In press.
21. Fujita, T., J. Iwasa and C. Hansch, J. Amer. Chem. Soc. 86:5175 (1964).
22. Hansch, C., and T. Fujita, Ibid. 86:1616 (1964).
23. Thorp, J.M., "Proceedings of the 13th Meeting of the European Society for the Study of Drug Toxicity," Berlin, 1971, p. 98.
24. Chang, Y.-H., R. Pinson, Jr. and M.H. Malone, Biochem. Pharmacol. 16:2053 (1967).
25. White, L.W., J. Pharmacol. Exp. Therap. 178:361 (1971).
26. Maragoudakis, M.E., J. Biol. Chem. 245:4136 (1970).
27. Musa, B.U., J.T. Ogilvie and J.T. Dowling, Metabolism 17:909 (1968).
28. McKerron, C.G., R.L. Scott, S.P. Asper and R.I. Levy, J. Clin. Endocrinol. Metab. 29:957 (1969).
29. Barbosa, J., and L. Oliner, Metabolism 18:141 (1969).
30. Fallon, H.J., L.L. Adams and R.G. Lamb, Lipids 7:106 (1972).
31. Segal, P., P.S. Roheim, and H.A. Eder, Circulation 3:(Suppl. 39) 182 (1969) (Abstract).

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Effect of Diet on Triglyceride Structure and Composition of Egg Yolk Lipids

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ABSTRACT

Hens were fed a practical diet supplemented and unsupplemented with 5% menhaden oil and a synthetic fat-free diet for a period of 90 days. Egg yolks from hens fed each of the three diets were analyzed for fatty acid composition and positional distribution of the fatty acids by successive chromatographic techniques. The triglycerides were resolved into fractions containing, 0, 1, 2 and 3+ double bonds per molecule. Each of these types was quantitated and analyzed for fatty acid distribution. The positional distribution was determined with the aid of pancreatic lipase hydrolysis. The feeding of the practical diet supplemented with 5% menhaden oil produced an increase in the 14:0 acid in the intact triglycerides, 2-monoglycerides and 1,3-diglycerides with the majority of this acid being bound in the 1,3 positions. In the monounsaturated triglycerides the 16:0 acid was linked predominantly at the 1,3 positions. The feeding of the fat-free diet produced a decrease in the 16:1 acid content of the egg yolk lipids in the monounsaturated series, in the intact triglycerides, the 2-monoglycerides and the 1,3-diglycerides. The 18:0 acid was linked more often at the 1,3 positions than at the 2 position, and was not affected by the diet consumed by the hens. Hens fed the fat-free diet produced more monounsaturated and diunsaturated triglycerides than those fed the other diets. Linoleic acid exhibited the greatest degree of preference for the 2 position, which was followed in turn by oleic acid. All other major acyl components were found to be preferentially esterified at the 1,3 positions. The difference observed in the fatty acid composition of egg yolk neutral and polar lipids was attributable to the fatty acid content of the diet. In the case of the oleic and linoleic acids, there was less variation in the saturated fatty acid content, which could be traced to the type of diet fed.

INTRODUCTION

The quantity of phospholipids, neutral fat, total lipids and cholesterol in egg yolk fat cannot be influenced by the type or quantity of fat in the diet of the hen (1-4). The fatty acid content of neutral fat and phospholipids can be influenced significantly by the fatty acid composition of the fat in the diet of the hen (1,3,5-11). The laying hen is incapable of synthesizing polyunsaturated fatty acids from nonfat precursors and is especially incapable of synthesizing linoleic acid (2,12). It has been shown that in vivo synthesis of linoleic acid may occur when a suitable precursor such as *cis*-2-octenoic acid is supplied in the diet through a pathway involving five elongations and a single desaturation step (13,14). Dietary linoleic and linolenic acids have been reported to be precursors of arachidonic (20:4), docosapentaenoic (22:5), eicosapentaenoic (20:5) and docosahexaenoic (22:6) acids, respectively (15). It has been shown that laying hens can synthesize eicosatrienoic (20:3) acid (2,16), probably through a desaturation and elongation of endogenous stearic acid (18:0) as has been suggested in the case of fat-deficient rats (17). It has been reported that, when hens were fed a practical diet with and without trielaidin, the saturated fatty acid occupied positions 1 and 3 preferentially in egg yolk triglycerides, whereas linoleic acid was found almost exclusively confined to the 2 position (18). Oleic acid was found to be abundant in all positions but perferentially distributed at the 2 position of the triglyceride molecule. In contrast, elaidic acid was found to be readily incorporated in the yolk triglycerides preferentially at the 1 and 2 positions. In another study on egg triglycerides (19) it was reported that ca. 66% of the yolk fat from hens fed a practical diet (PD) was triglycerides, which were found to contain 39% triunsaturated (UUU), 31% monosaturated, diunsaturated (SUU), 28% disaturated, monounsaturated (SSU) and 2% trisaturated fatty acids.

The purpose of the study reported herein was to investigate the positional distribution of chicken yolk triglycerides from hens fed a fat-free diet (FFD) and a PD, the latter supplemented with 5% menhaden oil (MO).

EXPERIMENTAL PROCEDURES

Thirty inbred hybrid pullets, 22 weeks of age, were selected and distributed at random into three groups of ten birds each in individual

TABLE I

Fatty Acid Composition of Lipids in Experimental Diets and of Menhaden Oil (wt %)[a]

Component	PD	PD + 5% MO	FFD	MO
% Ether extract	3.3	7.4	0.25	+[b]
14:0	2.8	6.1	2.9	11.8
16:0	17.7	24.7	22.3	26.4
16:1	3.3	9.8	4.6	13.4
18:0	5.6	4.4	9.8	4.0
18:1	21.2	17.2	30.1	12.3
18:2	39.8	16.9	26.7	2.1
18:3	4.9	2.4	2.0	2.4
20:5	—	8.0	—	12.0
21:0	—	1.8	—	2.5
22:1	2.7	0.7	—	1.1
22:5	—	1.5	—	2.0
22:6	—	2.9	—	4.4
24:1	—	0.6	—	1.2

[a]Abbreviations: PD, practical diet; MO, menhaden oil; FFD, fat-free diet.
[b]+, not determined.

wire cages. The first group was fed a diet composed of 21.46% ground yellow corn, 42.93% ground sorghum grain, 20.86%, soybean oil meal (44% protein), 2% menhaden fish meal, 3% dehydrated alfalfa meal, 6% oyster shell flour, 2.5% defluorinated rock phosphate, 0.25% sodium chloride and 1% of a vitamin trace mineral premix. The latter supplied the following per kilogram of diet: 8800 IU vitamin A, 2200 ICU vitamin D_3, 4.4 mg riboflavin, 11 mg *d*-calcium pantothenate, 27.5 mg niacin, 0.013 mg vitamin B_{12}, 5.5 IU vitamin E, 2.2 mg menadione sodium bisulfite, 126 mg ehtoxyquin, 22 mg bacitracin, 62.5 mg ZnO, 138 mg $MnSO_4$, 660 mg choline chloride and 50 mg 3-nitro-4-hydroxyphenylarsonic acid. The second group was fed the same diet supplied with 5% menhaden fish oil with the protein adjusted isonitrogenously. The third group was fed a FFD composed of the following: 65.75% glucose monohydrate, 18.88% isolated soybean protein, 6.00% oyster shell flour, 2.50% defluorinated rock phosphate, 0.25% salt, 1.12% wood pulp and 5.50% vitamin and mineral mix. The vitamin and mineral premix supplied the following per kilogram of diet: 10 mg thiamin, 10 mg riboflavin, 20 mg *d*-calcium pantothenate, 8 mg pyridoxine hydrochloride, 20 mg *p*-aminobenzoic acid, 500 mg inositol, 200 μg biotin, 75 mg niacin, 4 mg folic acid, 10,000 IU vitamin A, 4000 ICU vitamin D_3, 20 mg *d*-α-tocopherol acetate, 1500 mg choline chloride, 2.2 mg menadione sodium bisulfite, 0.020 mg vitamin B_{12}, 7.5 g methionine, 3.5 g glycine, 22 mg bacitracin, 126 mg ethoxyquin, 370 mg $MnSO_4$, 5.27 g $MgSO_4$, 150 mg $FeSO_4$, 11 mg $CuSO_4$, 104 mg $ZnCl_2$, 7 mg KI, 1.6 mg $CoCl_2$, 7.6 g KCl and 0.76 mg Na_2MoO_4. The birds were fed the diets indicated for a period of 90 days, at which time the representative sample of eggs were collected from each group for analysis.

Biochemical Procedures

The lipid material was extracted from the egg yolk samples by the procedure of Folch et al. (20) and fractionated by the silicic acid slurry method of Murty et al. (12). Yolk triglycerides were isolated from the neutral lipid fractionation by preparative thin layer chromatography according to the method described by Privett et al. (21). Yolk triglycerides were separated on the basis of the number of ethylenic linkages per triglyceride molecule according to the argentation procedures of Culp et al. (22) and Blank et al. (23). Fractionated egg yolk triglycerides were subjected to pancreatic lipase hydrolysis according to the method of Luddy et al. (24) for triglycerides containing one or more double bonds, and to the procedure of Barford et al. (25) for triglycerides containing only saturated components. The fatty acid moieties from the 2-monoglycerides that resulted from the enzymic lipolysis of triglycerides were analyzed by gas liquid chromatography. These data, in conjunction with the fatty acid composition of intact triglycerides, made it possible to estimate the fatty acid composition at the 1 and 3 positions according to the calculations of Vander Wal (26) and Coleman (27).

The fatty acid compositions of the yolk total and fractionated triglycerides, polar lipids and 2-monoglycerides from the enzymic hy-

drolysis of triglycerides, PD, menhaden oil and FFD were determined by gas liquid chromatography. Methyl esters of the fatty acid moieties were prepared according to the method of Feldman et al. (28) and analyzed as outlined by Saloma et al. (11). The total fat content of the PD, the PD + 5% MO and the FFD was determined according to the method outlined by the Association of Official Agricultural Chemists (29).

Data obtained from this study were statistically analyzed by the procedures of Snedecor (30). Significantly different means were separated through the use of Duncan's Multiple Range Test (31).

RESULTS AND DISCUSSION

The lipid content of the PD, PD + 5% MO and the FFD was 3.3, 7.4 and 0.25%, respectively (Table I). Linoleic acid was found to be present in the highest percentage in the PD. The second diet, PD + 5% MO, contained approximately the same quantity of linoleic acid when the fat content of the diet was taken into account. A negligible amount of linoleic acid was found in the FFD, considering that the total fat content was only 0.25%. The menhaden oil contained appreciable quantities of the polyunsaturated fatty acids (PUFA) that are normally found in fish fat: eicosapentanoic, docosapentanoic and docosahexanoic.

Distribution of Egg Yolk Triglyceride Types

There was little effect from adding 5% menhaden oil to the PD on the distribution of egg yolk triglyceride types (Table II). A decided difference existed in egg yolk triglyceride types from hens fed the FFD, especially in those triglycerides containing 2 and 3+ double bonds.

Fatty Acid Composition of Egg Yolk Triglycerides

The fatty acid content of the egg yolk triglycerides shown in Table III is given for the major fatty acid components found in the triglycerides. It should be pointed out that all of the other fatty acids were determined by gas liquid chromatography, as illustrated in Table I. Traces and quantities of 1% or less were eliminated in order to save tabular space.

The fatty acid composition of egg yolk triglycerides was altered by the diet that the hens received (Table III). Differences in dietary treatments did not alter the proportion of saturated fatty acids in yolk triglycerides significantly, with the exception of the 18:0 acid in the egg yolk triglycerides from hens fed the FFD. The fatty acid compositions of the yolk triglycerides from hens fed the PD and PD + 5% MO were found to be similar, although the value for the 18:1 acid was lower and that of the 18:2 acid slightly higher in the egg yolk triglycerides from hens fed PD + 5% MO. The 18:2 acid content of the egg yolk triglycerides from hens fed the FFD was decreased significantly to a value of less than 1%. These data are in agreement with earlier published reports, in that the fatty acid content of egg yolk triglycerides can be significantly altered by the fat content and the type of fat fed the laying hens (1,3,5-11). Attention is also directed to the fact that the egg yolk triglycerides from hens fed FFD were almost devoid of polyunsaturated fatty acids but contained the highest percentage of the 18:1 acid. From these data it may be stated that with the exception of the polyunsat-

TABLE II

Effect of Diet on Distribution of Egg Yolk Triglyceride Types in Chickens (wt %)[a]

Double bond no.[b]	Dietary treatment[c] PD	PD + 5% MO	FFD
0[d]	1.5★●	1.9★	1.0●
1	7.7●	10.1★●	15.3★
2	40.1●	40.9●	61.9★
3(+)	50.7★	47.1★	21.8●

[a]Mean of duplicate samples.

[b]Refers to number of double bonds per triglyceride molecule. Fractions resolved by Ag^+ thin layer chromatography.

[c]For abbreviations see Table I.

[d]Within each triglyceride fraction, means bearing different superscripts are significantly different ($P < .05$).

TABLE III

Effect of Diet on Fatty Acid Composition of Chicken Egg Yolk Triglycerides (wt %)[a]

Component[b]	Dietary treatment[c] PD	PD + 5% MO	FFD
16:0[d]	24.3	25.2	24.4
16:1	4.6	6.4	4.8
18:0	5.3●	6.6★●	9.1★
18:1	53.4★●	45.6●	59.5★
18:2	10.7★	12.2★	0.8●
% SFA[e]	30.5	33.7	34.0
% MUFA	58.0	52.0	64.3
% PUFA	11.2	14.1	1.4

[a]Mean of duplicate samples.

[b]Chain length; number of double bonds.

[c]For abbreviations see Table I.

[d]Within each component, means bearing different superscripts are significantly different ($P < .05$).

[e]S, saturated; MU, monounsaturated; PU, polyunsaturated; FA, two fatty acids.

TABLE IV

Effect of Diet on Positional Distribution of Fatty Acids in Chicken Egg Yolk[a]

	Trisaturated triglycerides[c]								
	Intact triglycerides			2-Monoglycerides[d]			1,3-Diglycerides[e]		
Component[b]	PD[c]	PD + 5% MO	FFD	PD	PD + 5% MO	FFD	PD	PD + 5% MO	FFD
14:0	3.6	5.6	2.0	4.3	6.4	4.0	3.3	5.3	0.9
16:0	56.0	54.0	55.2	48.2	49.6	48.5	60.0	56.2	58.5
18:0	40.4	40.4	42.8	47.5	44.0	47.5	36.7	38.5	40.6
				Monounsaturated triglycerides					
14:0	1.7	3.0	0.9	1.2	2.0	0.7	1.9	3.5	1.0
16:0	39.1	37.8	38.2	18.2	15.3	13.4	49.7	49.0	50.5
16:1	3.9	4.3	1.6	3.0	4.7	2.1	4.3	4.2	1.4
18:0	23.6	21.2	24.2	16.0	14.4	15.8	27.4	24.4	28.5
18:1	31.6	33.7	35.1	61.6	63.6	68.0	16.7	19.0	18.6
				Diunsaturated triglycerides					
16:0	25.0	26.6	27.6	3.5	2.8	5.6	35.7	38.3	38.6
16:1	4.0	6.1	4.1	3.2	5.2	3.7	4.4	6.5	4.3
18:0	5.9	7.5	7.9	2.8	2.2	5.4	7.4	10.2	9.1
18:1	57.0	53.8	59.6	71.0	76.0	83.6	50.0	42.4	47.1
18:2	7.7	5.0	tr	19.0	13.3	tr	2.0	1.1	tr
				Triglycerides containing 3+ double bonds					
16:0	12.8	17.4	8.5	2.9	5.1	4.8	17.7	23.5	10.2
16:1	7.2	8.0	9.3	2.0	5.0	3.9	9.8	10.5	12.0
18:0	2.8	3.0	2.0	2.0	2.4	3.4	3.2	3.4	1.3
18:1	47.0	41.0	72.9	31.0	30.5	79.1	54.8	46.6	69.8
18:2	27.0	23.6	3.4	57.0	52.0	5.5	11.9	10.0	2.4
18:3	2.0	3.0	1.0	3.1	2.4	0.7	2.0	3.1	1.1

[a]Mean of duplicate samples.
[b]Chain length: number of double bonds.
[c]PD, practical diet; MO, menhaden oil; FFD, fat-free diet; tr, trace (< 0.4%).
[d]Obtained by pancreatic lipase hydrolysis of intact triglycerides.
[e]Calculated from fatty acid compositions of intact triglycerides and 2-monoglycerides.

urated fatty acids, and more specifically, 18:2, the synthesis of yolk triglycerides may proceed normally, even under conditions of negligible fat intake by the hen.

Trisaturated Triglycerides

The addition of 5% menhaden oil to the PD produced an increase in the 14:0 content of the trisaturated triglycerides in egg yolk lipids of the intact triglycerides, the 2-monoglycerides and the 1 plus 3-glycerides (Table IV). The 14:0 acid content of egg yolk lipids from hens fed the FFD was decreased in the intact triglycerides. Apparently the majority of the 14:0 acid was concentrated in the 2-monoglyceride fraction, since there was only 0.9% of this acid in the 1 plus 3 position of egg yolk lipids from hens fed the FFD. Diet had little effect on the 16:0 content of egg yolk lipids in the intact triglycerides, 2-monoglycerides and 1 plus 3-diglycerides, with the possible exception of the PD + 5% MO fed birds. There was a tendency for the 18:0 acid to occupy the 2-monoglyceride position in the intact 2-monoglycerides, irrespective of diet fed in the saturated triglycerides.

Monounsaturated Triglycerides

The major components found in the monounsaturated triglycerides are the 16:0, 18:0 and 18:1 acids (Table IV). Palmitic and stearic acids appeared in virtually equimolar proportions at the secondary alcoholic position. The 16:0 acid was more abundant than the 18:0 at the 1 plus 3 positions, whereas the 18:1 acid was preferentially acetylated at the 2 position.

There was an increase in the 14:0 acid in the monosaturated triglycerides when the hens were fed PD + 5% MO in the intact triglycerides, 2-monoglycerides and 1 plus 3-diglycerides, with the majority of the 14:0 being bound in the 1 plus 3 positions. In the monounsaturated triglycerides, there was a tendency for the 16:0 acid to be linked to the 1 plus 3-diglycerides more than at the 2 position. In the monounsaturated triglycerides, there was a tendency for the 16:0 acid to be linked to the 1 plus 3-diglycerides more than at the 2 position. The feeding of the FFD produced the lowest content of the 16:0 at the 2 position. The feeding of the FFD to the hen produced a decrease of the 16:1 acid in the monosaturated series in the intact triglycerides, the 2-monoglycerides and the 1 plus 3-diglycerides. The 18:0 acid tended to be linked to the 1 plus 3 positions more than to the 2 position in the monounsaturated triglycerides, with little effect traceable to the diet consumed by the hens. The feeding of the FFD to the hens produced an increase in the 18:1 acid in the intact triglycerides of the monosaturated series, with this acid showing the highest affinity for the 2 position. There was a predominance of the 18:1 acid to occupy the 2-monoglyceride position in constrast to the 1 plus 3 positions, irrespective of the diet in the monounsaturated triglyceride series.

Diunsaturated Triglycerides

It is very apparent that the 16:0 acid had a low affinity for the 2 position in the diunsaturated triglyceride series of the egg yolk lipids (Table IV). There was little effect from diet on the 16:0 content of the intact triglycerides, 2-monoglycerides and 1 plus 3-diglycerides in this triglyceride series. The feeding of the PD + 5% MO increased the 16:1 acid content of intact triglycerides in the diunsaturated triglyceride series that was apparent in the 2-monoglycerides and 1 plus 3-diglycerides. The feeding of the PD + 5% MO and the FFD produced egg yolk lipids with an increased level of 18:0 in the intact triglycerides, 2-monoglycerides and 1 plus 3-diglycerides of the diunsaturated series. The feeding of the PD + 5% MO produced a decrease in the 18:1 acid content of the intact triglycerides, whereas the FFD produced an increase in the 18:1 acid content of the intact triglycerides of the diunsaturated series. There was a decided affinity in the egg yolk lipids from hens fed the PD + 5% MO and the FFD for the 18:1 acid to occupy the 2 position. Apparently there is some characteristic in the menhaden oil that causes the 18:1 acid to be linked at the 2 position in this series. It is understandable that the 18:1 acid would be increased in the 2 position of egg yolk lipids from hens fed the FFD, since there is little or no 18:2 acid to compete for this position in the diunsaturated triglycerides. It may be noted further that there was a decrease in the 18:1 acid in the 1 plus 3-diglycerides of the diunsaturated series. The feeding of the PD or PD + 5% MO produced an increase in the 18:2 acid content of the egg yolk lipids, with the majority of the 18:2 acid located at the 2 position in the diunsaturated triglycerides. Only a trace of the 18:2 acid was found in egg yolk lipids from hens fed the FFD.

Triglycerides Containing Three or More Double Bonds

In the egg yolk lipid triglycerides containing 3+ double bonds, the feeding of PD + 5% MO produced an increase in the 16:0 acid in the intact triglycerides, the 2-monoglycerides and the 1 plus 3-diglycerides (Table IV). The 16:0 acid appeared to occupy the 1 plus 3 positions to a greater extent than the 2 position. There

TABLE V

Calculated Composition of Egg Yolk Triglycerides from Hens Fed Various Diets[a]

Triglyceride species[b]	Diet, % w/w[c]		
	PD	PD + 5% MO	FFD
SSS	1.5	1.9	1.0
SSM	2.7	3.2	4.6
SMS	5.0	6.9	10.7
SMM	29.9	33.2	7.2
MSM	2.5	2.3	54.7
SDS	7.7	5.4	—
Others	50.7	47.1	21.8

[a]Calculated according to Blank et al. (23).

[b]S, M and D indicate saturated, monoenoic and dienoic acids, respectively.

[c]For abbreviations see Table IV.

was little effect of diet on the position of the 16:1 and 18:0 acids in the triglycerides containing 3+ double bonds. The most dramatic changes traceable to diet was found in the 18:1 and 18:2 acid content of the egg yolk triglycerides containing 3+ double bonds. There was a very significant increase in the 18:1 acid egg yolk triglycerides containing 3+ double bonds when the hens were fed a FFD. This increase persisted in the location of the fatty acids at the 1 plus 3 position but was greatest at the 2 position. When the laying hen is provided with linoleic acid, this acid occupies the 2 position to a predominant extent in egg yolk triglycerides containing 3+ double bonds, and occupies the 1 plus 3 positions to a much lesser extent. When a FFD is fed, the linoleic acid content of egg yolk triglycerides containing 3+ double bonds is quite low, with the majority of the 18:2 acid chains occupying the 2 position. The 18:3 acid was decreased in egg yolk triglycerides from hens fed the FFD and appeared to have somewhat more of an affinity for the 2 position than for the 1 plus 3 positions. When the laying hen is not provided with the 18:2 acid, as in the case of those fed the FFD, the 18:1 acid predominates at the 2 position in egg yolk triglycerides containing 3+ double bonds.

From the above it can be stated that diet has little effect on the fatty acid composition of trisaturated triglycerides. The primary effect of diet may be noted in the monounsaturated triglycerides and especially in the diunsaturated triglycerides. In the monounsaturated triglycerides there is a tendency for the 18:1 content to increase when menhaden oil is included in the diet at the 5% level and when the FFD is fed. The primary fatty acids attached at the 2 position are 18:1 and 18:2 in the diunsaturated triglycerides. The 18:1 content is increased in

TABLE VI

Effect of Diet on Fatty Acid Composition of Chicken Egg Yolk Polar Lipids (wt %)[a]

Component[b]	Dietary treatment[c]		
	PD	PD + 5% MO	FFD
16:0[d]	25.0●	28.1★	25.2●
16:1	2.1●	3.3★	2.7★●
18:0	16.5	13.9	15.4
18:1	27.2●	24.6●	42.2★
18:2	15.0★	11.6●	2.7■
20:4	8.0★	2.7■	4.0●
20:5	—	1.8	—
22:5	—	1.4	—
22:6	2.9●	11.8★	1.4●
% SFA[e]	43.0	42.7	42.9
% MUFA	30.7●	27.9●	45.5★
% PUFA	26.3★	29.4★	11.6●

[a]Mean of duplicate samples.

[b]Chain length: number of double bonds.

[c]For abbreviations see Table IV.

[d]Within each component, means bearing different superscripts are significantly different ($P < .05$).

[e]S, saturated; MU, monounsaturated; PU, polyunsaturated; FA, fatty acids.

egg yolk lipids from hens fed a diet containing 5% menhaden oil or those fed the FFD. The 18:2 content of egg yolk lipids is definitely influenced by diet, was highest at the 2 position of egg yolk lipids from hens fed the PD and was decreased when 5% menhaden oil was added to the diet, wtih only a trace appearing in lipids from hens fed the FFD. The menhaden oil has a direct influence in increasing the 18:1 and decreasing the 18:2 at the 2 position in the diunsaturated triglyceride series. Thus it would appear from these data that the 18:1 and 18:2 acids are predominantly attached in the 2 position in the monounsaturated and diunsaturated triglycerides. The 18:1 acid is linked predominantly to the 2 position when hens are fed the FFD in egg yolk triglycerides containing 3+ double bonds. If the hen is provided a dietary source of the 18:2 acid, this acid occupies predominantly the 2 position in those triglycerides containing 3+ double bonds.

These data are in agreement with a previous report (32) in which it was shown that saturated fatty acids occupied positions 1 plus 3, whereas 18:2 was almost exclusively confined to the 2 position in egg yolk triglycerides. This report also stated that the 18:1 acid was found to be abundant at all positions but was preferentially distributed at the 2 position.

Triglyceride Analysis

Diet had no effect on the saturated egg yolk triglycerides and little effect on the disaturated monoenoic triglycerides (Table V). When the

monoenoic acid occupied the β position and the saturated acids the α,α' positions, there was an increase in this egg yolk triglyceride from hens fed the FFD. When hens were fed the PD or PD + 5% MO, there was a definite increase in the triglyceride containing the monoenoic acid in the β position, and a saturated and a monoenoic acid in the α,α' positions. When the triglyceride contained a saturated fatty acid in the β position, and monoenoic acids in the α,α' positions, there was a definite increase in the triglyceride resulting from the feeding of the FFD. Appreciable quantities of the triglyceride containing saturated fatty acids in the α,α' positions and a dienoic acid in the β position were found in egg yolks from hens fed PD or PD + 5% MO. This triglyceride was not detected in egg yolk lipids from hens fed FFD. These data are in agreement with the report of Privett et al. (36) in studies with rat tissue, which showed that the amount of each type of triglyceride varied considerably in relation to the fatty acid composition of the diet.

Fatty Acid Composition of Yolk Phosphatides

There was no dietary effect from feeding PD, PD + 5% MO, or FFD on the 16:0, 16:1 or 18:0 fatty acid content of the egg yolk polar lipids (Table VI). Hens fed the PD and PD + 5% MO produced egg yolk phosphatides that contained approximately the same quantity of the 18:1 acid; those fed the FFD had a significantly higher value for this acid. There was a significant decrease in the 18:2 acid content of egg yolk phospholipids from hens fed the PD + 5% MO and that of those fed the PD. The 18:2 acid content of egg yolk phosphatides from hens fed the FFD was very significantly decreased below that of the groups fed the PD and PD + 5% MO. The 20:4 acid content of egg yolk polar lipids from hens fed the PD was significantly higher than that of those fed the PD + 5% MO. The egg yolk polar lipid content from hens fed the FFD was significantly higher in 20:4 acid than that of those fed PD + 5% MO. The egg yolk polar lipids from the hens fed PD + 5% MO contained 1.8% of the 20:5 acid and 1.4% of the 22:5 acid. A significantly high value was recorded for the 22:6 acid in lipids from hens fed the PD + 5% MO. It is possible that the hens converted 20:5 to 22:6, a conversion that would not be unreasonable in light of the evidence existing that the 20:5 and 22:6 in oils from marine species belong to the ω-3 family (33,34). It was obvious from these data that the low level of linoleic acid in the FFD affected the deposition of this acid in the egg yolk polar lipids. It is also apparent that the 18:1 acid was synthesized to a considerable degree by hens fed FFD. The distribution of the fatty acids in the egg yolk phosphatides was not determined, since such data have been published previously (18,19,21,35).

REFERENCES

1. Reiser, R., J. Nutr. 40:429 (1950).
2. Reiser, R., Ibid. 44:159 (1951).
3. Fisher, H., and G.A. Leveille, Ibid. 63:119 (1957).
4. Ostrander, J.G., R. Jordan, W.J. Stadelman, J.C. Rogler and G.E. Vail, Poultry Sci. 39:746 (1960).
5. Evans, J.E., S.L. Bandemer and J.A. Davidson, Ibid. 39:1199 (1960).
6. Evans, J.E., J.A. Davidson and S.L. Bandemer, J. Nutr. 73:282 (1961).
7. Evans, J.E., J.A. Davidson, J.N. LaRue and S.L. Bandemer, Poultry Sci. 42:875 (1963).
8. Leveille, G.A., and H. Fisher, Ibid. 37:658 (1958).
9. Feigenbaum, A.S., and H. Fisher, Ibid. 37:1203 (1958).
10. Machlin, L.J., R.S. Gordon, J. Marr and C.W. Pope, Ibid. 41:1340 (1962).
11. Saloma, A.E., F. Farr, J. Cooper and J.R. Couch, Nutr. Rep. Int. 3:5,263 (1971).
12. Murty, N.L., M.C. Williams and R. Reiser, J. Nutr. 72:451 (1960).
13. Reiser, R., N.L. Murty and H. Rakoff, J. Lipid Res. 3:56 (1962).
14. Murty, N.L., H. Rakoff and R. Reiser, Biochem. Biophys. Res. Commun. 8:372 (1962).
15. Murty, N.L., and R. Reiser, J. Nutr. 75:287 (1961).
16. Miller, E.C., H. Menge and C.A. Denton, Ibid. 80:431 (1963).
17. Mead, J.F., and W.H. Slaton, Jr., J. Biol. Chem. 219:705 (1956).
18. Kuksis, A., and L. Marai, Lipids 2:217 (1967).
19. Privett, O.S., and L.J. Nutter, Ibid. 2:149 (1967).
20. Folch, J., M. Lees, G.H. Sloane-Stanley, J. Biol. Chem. 226:497 (1957).
21. Privett, O.S., M.L. Blank and J.A. Schmit, J. Food Sci. 27:463 (1962).
22. Culp, T.W., R.D. Harlow, C. Litchfield and R. Reiser, JAOCS 42:974 (1965).
23. Blank, M.L., B. Verdino and O.S. Privett, Ibid. 42:87 (1965).
24. Luddy, F.E., R.A. Barford, S.F. Herb, P. Magidman and R.W. Riemenschneider, Ibid. 41:693 (1964).
25. Barford, R.A., F.E. Luddy and P. Magidman, Lipids 1:287 (1966).
26. Vander Wal, R.J., "Advances in Lipid Research," Vol. 2, Academic Press, Ind., New York, 1964, p. 1.
27. Coleman, M.H., JAOCS 38:685 (1961).
28. Feldman, G.L., H.T. Johnson, T.W. Culp, R.H. Gowan, Poultry Sci. 41:1851 (1962).
29. "Official Methods of Analysis of the Association of Official Agricultural Chemists," Edited by W. Horwitz, Ninth edition, AOAC, Washington, D.C., 1960, p. 287.
30. Snedecor, G.W., "Statistical Methods," Fifth edition, Iowa State University Press, Ames, Iowa, 1965, p. 237.
31. Duncan, D.B., Biometrics, 11:1 (1955).
32. Leclercq. B., A. Agot and J.C. Blum, Academiedes Sciences, Comptes Rendus 262:2540 (1966).
33. Bottino, N.R., G.A. Vandenburg and R. Reiser, Lipids 2:489 (1967).

34. Ackman, R.G., C.A. Eaton and P.M. Jangaard, Can. J. Biochem., 43:1513 (1965).
35. Tattrie, N.H., J. Lipid Res. 1:60 (1959).
36. Privett, O.S., M.L. Blank and B. Verdino, J. Nutr. 85:187 (1965).

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Preferential Labeling of Phosphatidylcholine during Phospholipid Synthesis by Bovine Mammary Tissue

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ABSTRACT

Bovine mammary cells and tissue synthesize de novo the classes of phospholipids, found in mammary tissue and milk, from various precursor molecules. Several short term experiments were carried out in vitro, using labeled precursors, i.e., 1-^{14}C-fatty acids; 2-^{14}C-acetate; U-^{14}C-glycerol; 1,2-^{14}C-choline; 1,2-^{14}C-ethanolamine; 2-^{14}C-serine; and Me-^{14}C-methionine. All the phospholipid classes were labeled. The specific activity of tissue phosphatidylcholine was consistently three to six-fold greater than that of phosphatidylethanolamine. The results indicated that stepwise methylation of phosphatidylethanolamine with labeled methyl group of methionine was occurring to a minor extent, as was a negligible amount of choline exchange. Serine was incorporated into phosphatidylserine and sphingomyelin. Significant quantities of labeled phosphatidylserine were decarboxylated to phosphatidylethanolamine. Apparently phosphatidylcholine was synthesized de novo from choline via phosphorylcholine and CDP-choline. Based on the present observations and other data, it is suggested that there may be two pools of phosphatidylcholine in lactating mammary cells.

Phospholipids are secreted in bovine milk as phospholipoproteins complexed in the fat globule membrane and as discrete lipoprotein particles (1-19). A bovine mammary gland yielding 25 liters milk secretes ca. 10 ± 3 g phospholipids per day which corresponds to an average 5% of the mammary tissue phospholipids. The various phospholipid (PL) classes are secreted in different amounts. Approximately equal amounts of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are secreted in bovine milk (14,15,20). Based on their respective tissue concentrations, PE is expected to have a more rapid turnover rate than PC because its intracellular concentration is ca. 50% that of PC (14,15,17). However in vivo and in vitro studies using labeled fatty acids, glycerol and ^{32}P reveal that PC is the most rapidly labeled mammary and milk PL (17-19, 21-25). Because these results may be explained by the activity of a number of different biosynthetic routes or possibly exchange reactions and because in vivo studies may be confounded by possible extramammary PL precursors (though evidence is to the contrary [25]), studies with mammary tissue have been made to compare the relative rates of PC and PE labeling when radioactive

TABLE I

Incorporation of Long Chain Fatty Acids into Phospholipids by Dispersed Mammary Cells in Vitro[a]

	Incorporation (cpm x 10^3)							
	Cultured tissue				Culture medium			
	Incubation time, hr							
Substrate	1.5	3	6	12	1.5	3	6	12
Sodium-1-^{14}C-myristate	—	115.2	—	134.4	—	19.2	—	41.6
Sodium-1-^{14}C-palmitate	148.8	481.6	740.8	803.2	8.0	14.4	30.4	48.0
Sodium-1-^{14}C-stearate	144.0	224.0	505.6	624.0	4.8	11.2	22.4	24.0
Sodium-1-^{14}C-oleate	352.0	454.4	480.0	528.0	16.0	27.1	20.8	35.2

[a]Mammary cells, equivalent to 5.0 mg protein, were incubated with 40 μM of the sodium salts of each fatty acid (10μCi/mol). Lipids were isolated as described in methods.

TABLE II

Percentage Distribution of Radioactivity in Cellular Phospholipid Classes following Incubation of Mammary Cells with Labeled Fatty Acids (Experiment I)[a]

Phospholipid class	Percentage distribution											
	Myristic-1-^{14}C		Palmitic-1-^{14}C			Stearic-1-^{14}C			Oleic-1-^{14}C			
	Incubation period, hr											
	3	12	1.5	3	6	12	1.5	3	6	12	1.5	3	6	12
Lysophosphatidylcholine	4.2	2.4	2.1	2.8	1.3	0.9	0.3	0.4	0.2	0.2	tr	tr	0.8	0.9
Sphingomyelin	4.9	4.8	3.1	4.2	4.6	5.0	1.0	1.8	2.1	3.5	0.3	0.2	0.6	1.0
Phosphatidylcholine	78.1	80.0	62.8	61.8	62.1	68.5	65.6	64.0	69.7	70.0	71.8	73.9	74.0	76.1
Phosphatidylinositol	4.5	3.2	10.2	9.7	8.0	6.2	18.0	17.0	11.0	8.1	17.1	13.8	11.6	8.3
Phosphatidylserine	tr[b]	tr	2.1	2.3	2.4	2.6	2.0	2.8	2.5	3.0	1.3	1.8	2.1	2.3
Phosphatidylethanolamine	6.4	7.0	16.8	17.0	16.2	15.4	12.1	12.0	13.3	14.1	8.5	9.0	9.7	10.3
Others	1.9	2.6	2.9	2.2	1.4	1.4	1.0	1.2	1.2	1.1	1.0	1.3	1.2	1.1

[a]Phospholipids were separated by two dimensional thin layer chromatography (14). Lipid spots were transferred to scintillation vials and radioactivity determined as described (17).

[b]tr = trace.

precursors were used in vitro.

MATERIALS AND METHODS

Mammary cells from lactating cows were dispersed with collagenase and cultured under aseptic conditions as described previously (26,27). Each treatment contained ca. 5 x 10^7 cells, equivalent to 5.0 mg protein in 6 ml culture medium. The appropriate radioisotopes in sterile buffer solution were added to the culture flasks after dispensing the cells, and the stoppered flasks were incubated for specified periods at 37 C. After incubation the cells were separated from the culture media by gentle centrifugation (300 g for 10 min). The lipids were extracted by the procedure of Folch et al. (28). The lipids were fractionated by two dimensional thin layer chromatography (14). Lipid phosphorus was quantified by the method of Rouser et al. (29). Argentation chromatography was used to separate PC species according to degree of unsaturation (30).

The radioactivity in the lipid spots, identified from known standards (Applied Science Lab., State College, Pa.), was determined by liquid scintillation spectrometry as reported previously (21). Cellular protein was quantified by a microkjeldahl method.

Radiochemically pure (99%) substrates were purchased from New England Nuclear (Boston, Mass.). Chromatographic supplies were obtained from Brinkmann (Westbury, N.Y.) and redistilled solvents were used.

RESULTS

Experiment I. Fatty Acid Incorporation

The fatty acids commonly occurring in bovine milk PL were incubated with the cell preparations as their respective sodium salts and incorporation into phospholipids of tissue and culture media were monitored (Table I). Incorporation of radioactivity into cellular PL increased markedly with time, and labeled PL accumulated in the media also. Though oleic acid ($C_{18:1}$) was incorporated most rapidly initially, the saturated acids palmitic ($C_{16:0}$) and stearic ($C_{18:0}$) were ultimately esterified to a greater extent. The ratio of radioactivity in the media PL showed that, compared to the intracellular PL there was a preferential secretion of PC, especially that labeled with $C_{14:0}$ and $C_{16:0}$. Analysis of the phospholipids revealed that the preponderance of the radioactivity was in the PC (Table II), though all of the PL classes were labeled in all experiments. The respective radioactivities and specific activities (SA) of cellular PC and PE (Table III) varied

TABLE III

Ratios of Radioactivity and Specific Activities of Phosphatidylcholine and Phosphatidylethanolamine Isolated from Mammary Tissue and Culture Media following Incubation with Labeled Fatty Acids (Experiment I)

Source	Ratio of radioactivity, PC/PE (cpm x 10^3)[a]													
	1-^{14}C-Myristic		1-^{14}C-Palmitic				1-^{14}C-Stearic				1-^{14}C-Oleic			
	Incubation time, hr													
	3	12	1.5	3	6	12	1.5	3	6	12	1.5	3	8	12
Cultured tissue	12.2	11.4	3.7	3.6	3.8	4.5	5.4	5.3	5.2	5.0	8.4	8.2	7.7	7.5
Culture media	21.9	15.0	9.4	8.8	11.4	14.0	5.4	5.6	5.6	5.1	—	4.6	—	5.2
Ratio of specific activity, PC/PE[b]	5.7	5.0	1.7	1.5	1.5	1.4	2.4	2.3	2.	2.1	3.6	3.6	3.0	3.3

[a]PC, phosphatidylcholine; PE, phosphatidylethanolamine.
[b]Specific activity expressed as cpm x $10^3/\mu$g phosphorus in specific phospholipid.

with each fatty acid substrate and the disparity in SA from $C_{14:0}$ and $C_{18:1}$ substrates was most marked. The SA ratio decreased with duration of each incubation and in general was lower than those obtained in subsequent experiments using other labeled substrates.

Experiment II. Acetate

The fatty acid labeling data were consistent with observations from in vivo experiments (19). Because of possible differences in intracellular location of PC and PE synthesizing enzymes and fatty acid activation, the relative extent of labeling of PC and PE by fatty acids of endogenous origin was examined (Table IV). Incorporation into both classes increased markedly with time but considerably more labeled fatty acids were acylated into the PC as shown by the SA ratios, which decreased with time. The SA ratios were similar to those obtained in experiment I for myristic acid.

Experiment III. Glycerol

Because the apparent preferential labeling of PC with exogenous and endogenous fatty acids could have been explained by active acyl transferase(s), the cells were incubated with labeled glycerol which was actively incorporated into the phosphoglycerides (Fig. 1). Initially PC contained four-fold more activity than the PE, but this difference decreased with duration of incubation. The SA of PE gradually increased with time, as observed in experiments I and II.

Experiment IV. Choline, Ethanolamine, Serine, Methionine

The above data unequivocally indicated that de novo labeling and probably net synthesis of PC exceeded that of PE. The possibility existed that PE was actually being synthesized within these cells but was rapidly being transformed to PC by transmethylation. To study this the cells were incubated with the series of different PL precursors listed in Table V. Significantly more choline was incorporated compared to the ethanolamine in all experiments. Methionine and serine were utilized in small quantities. Differences in utilization were observed between preparations; however the patterns of incorporation of the various precursors were consistent. Evidence of molecular interconversions, i.e., decarboxylation of phosphatidyl-

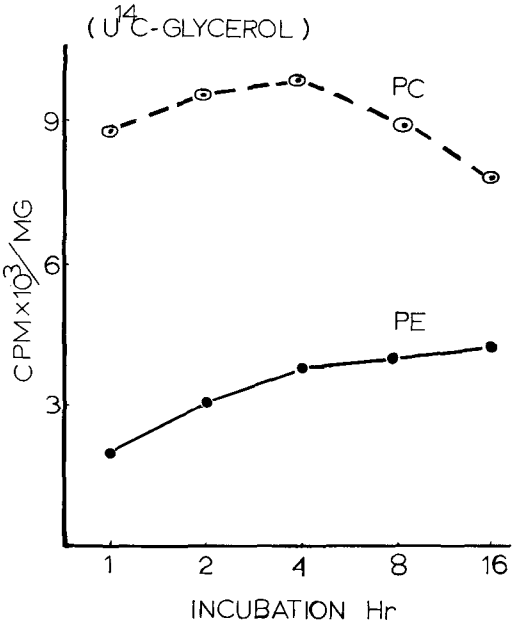

FIG. 1. Relative incorporation of U-^{14}C-glycerol into phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of mammary cells. Approximately 5 x 10^7 cells were incubated with 50 μM of U-^{14}C-glycerol (8mc/mol) for specified periods and lipids then analyzed (Experiment II). Specific activity expressed as cpm x 10^3/mg phospholipid.

TABLE IV

Differential Incorporation of Radioactivity into Cellular Phospholipids following Incubation of Bovine Mammary Cells with Na-2-^{14}C-Acetate (Experiment II)[a]

	Incorporation (cpm x 10^3)			
	Incubation period, hr			
Lipid class	2	4	8	24
Total phospholipids	37.0	126.0	256.0	645.0
Phosphatidylcholine (PC)	21.0	80.6	174.1	451.5
Phosphatidylethanolamine (PE)	1.6	7.5	15.3	35.0
Ratio of specific activity, PC/PE	5.2	4.5	4.6	4.1

[a]Tissue dispersions (ca. 5 x 10^7 cells) were incubated with 50 μM Na-2-^{14}C-acetate (specific activity ca. 10mc/nmol) in each treatment. Lipids were analyzed as described in Methods.

serine (PS) to PE, and methylation of PE to PC was obtained (Table VI). The preponderance of the ^{14}C-methyl group of methionine utilized was incorporated into the choline moiety of PC. The serine was incorporated mostly into PS through measurable quantities of the radioactive carbon were transformed into sphingomyelin, PE and some into neutral lipids. These data revealed that while stepwise methylation of PE was occurring in mammary cells, it was small compared to the rate of incorporation of choline into PC and probably could not account for the differential rate of labeling of PC and PE in these mammary cells when common precursors were used as substrates.

Experiment V. Choline Exchange

In the previous experiments the relatively greater incorporation of labeled choline into PL could perhaps be attributed to active base exchange within the cells, especially in the PC molecules. However the importance of this mechanism became doubtful following a series of experiments in which cells that had been incubated for specific times with labeled choline to attain a determined PC specific activity were then reincubated in media containing an excess of cold choline (40 mM), and changes in SA of choline phospholipids were monitored (Table VII). As in previous experiments the ^{14}C-choline was incorporated linearly with time, mostly into the PC (A-D). Incubation of the cells containing ^{14}C-choline labeled PC with excess choline revealed little evidence of exchange in the PC except between the 6 and 12 hr interval, when SA decreased, i.e., C compared to C^1. The SA of the lysoPC from cellular lipids decreased after the 3 and 6 hr incubations, indicating some loss of labeled choline or acylation of lysoPC to PC. The small decline in SA of the cellular PC has been observed in subsequent experiments and indicates that, while choline exchange may occur, the magnitude of this exchange in bovine mammary cells does not explain the apparent preferential synthesis of PC. The data in Table VII indicated that labeled PL of high SA was secreted into the culture media in these experiments, especially in the initial periods. These data indicated that de novo PC synthesis was occurring in these cells, apparently via the classical enzymatic pathway (31).

TABLE V

Incorporation of Different Radioactive Precursors into Phospholipids by Bovine Mammary Tissue in Vitro (Experiment IV)[a]

	Radioactivity incorporated (cpm x 10^3)			
	Substrate			
Incubation, hr	1,2-^{14}C-Choline	1,2-^{14}C-Ethanolamine	Me-^{14}C-Methionine	3-^{14}C-Serine
1	21.5	3.1	0.40	1.8
3	48.1	10.2	0.60	3.9
6	78.3	18.9	0.89	6.2
12	194.6	65.7	2.20	14.6

[a]Each treatment contained ca. 5 x 10^7 cells and 50 μM labeled substrate. Specific activities of substrates were 1,2^{14}C-choline and 1,2^{14}C-ethanolamine 2μCi/μmol; Me-^{14}C-methionine and 3-^{14}C-serine; 5μCi/μmol. Average data from two experiments.

Analysis of the distribution of the radioactivity in the various molecular species of cell PC following incubation of mammary cells with choline showed that the PC molecules containing two double bonds contained the greatest quantity of incorporated choline (Table VIII). The labeling in the monounsaturated PC molecules decreased, whereas that in the triunsaturated species increased with incubation time.

DISCUSSION

The utilization of free fatty acids by lactating mammary tissue for synthesis of glycerolipids both in vitro and in vivo has been well documented (19,22,32-36). Most of the labeled fatty acids associated with the phospholipids were acylated in PC (18,19,22). Endogenous fatty acids were mostly acylated into secretory triglycerides (27,32-37); however those in the phospholipids, i.e., myristic and palmitic acid, are preponderantly found in the PC class (34,35). The preferential labeling of PC may be explained by very active acyl transferase enzyme(s), which have a high specificity for choline phosphatides. These are very active in the plasma membrane of liver cells (38). While these enzymes are active in mammary tissue (Gross and Kinsella, unpublished data), their class specificity has not been determined and the present data would preclude this mechanism as accounting for the greater labeling of PC in all experiments. The preferential incorporation of glycerol into PC compared to PE by bovine tissue has been reported and discussed (21).

The disparity between PC and PE labeling was also observed when labeled choline and ethanolamine were used as substrates. In the present studies stepwise methylation of PE to PC using the methyl group of methionine (39-44) was negligible. This is a major pathway in rat liver (42). The conversion of PS to PE and of some PE to PC has not been reported in ruminant mammary tissue. Presumably these interconversions occur via the mechanisms of decarboxylation and stepwise methylation (31,41,43). Direct base exchange of choline with the choline of PC was apparently quite limited, even in the presence of excess choline. This was consistent with reports for other mammalian tissues, e.g., liver (45), intestine (46) and cultured mast cells (47), and indicated that the CDP-choline pathway was operating (31.48).

The variation in distribution of the ^{14}choline among PC molecular species in mammary cells may indicate that these have different turnover rates, as shown for PC species in several animal tissues (43-45, 49-51). The mo-

TABLE VI

Percentage Distribution of Radioactivity in Phospholipids following Incubation of Bovine Mammary Tissue with Labeled Precursors (Experiment IV)

Lipid class[a]	Substrate: 1,2-^{14}C-Choline				Me^{14}C-Methionine				1-2-^{14}C-Ethanolamine				3-^{14}C-Serine			
	Incubation period, hr: 1	3	6	12	1	3	6	12	1	3	6	12	1	3	6	
LYL	2.3	2.6	3.5	2.3	3.3	2.8	2.9	1.9	0.6	0.2	0.3	0.3	---	0.4	0.4	
SPH	0.7	1.0	2.5	3.9	1.9	2.3	1.9	1.7	0.7	0.5	0.4	0.8	10.4	11.8	10.9	
PC	96.3	96.2	94.0	93.8	70.5	74.3	83.0	89.1	6.8	2.6	4.6	5.9	3.0	2.0	1.3	
PS	---	---	---	---	---	---	---	---	---	---	---	---	72.7	57.4	52.6	
PE	---	---	---	---	1.1	1.3	1.2	0.3	89.9	93.6	91.9	91.0	6.9	20.9	25.3	
MPE	---	---	---	---	23.2	19.3	11.2	7.0	1.0	0.6	0.8	0.9	---	---	---	
NL	0.7	0.2	---	---	---	---	---	---	1.0	1.5	2.0	1.1	7.0	7.5	9.5	

[a] Abbreviations: LYL, lysophosphatidylcholine; SPH, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; MPE, methylated phosphatidylethanolamine; NL, neutral lipids.

TABLE VII

Specific Activities of Phospholipids Isolated from Cells and Culture Medium following Incubation of Bovine Mammary with 1,2^{14}C-Choline and an Excess of Choline (Experiment V)[a]

	Specific activity (cpm x 10^3/μg lipid P.)						
	Incubation time, hr						
	1	3	6	12	3	6	12
	Flask						
Lipid class	A	B	C	D	A^1	B^1	C^1
Total phospholipids	0.67	1.83	2.67	3.98	0.95	1.90	2.55
Cell phospholipids	0.66	2.30	3.92	6.22	1.04	2.44	3.28
Medium phospholipids	0.68	1.36	1.43	1.75	0.87	1.39	1.82
Cell lipids							
Phosphatidylcholine	1.36	5.16	8.08	18.01	1.77	5.73	7.32
Lysophosphatidylcholine	0.25	0.53	1.10	1.15	0.29	0.37	0.57
Sphingomyelin	0.10	0.56	0.83	0.95	0.22	0.36	0.23

[a]All flasks, containing 5 x 10^7 cells and ca. 50μM 1,2-^{14}C-choline (2 mc/mM), were initiated simultaneously. Flasks denoted A, B, C, D were terminated at 1,3,6,12 hr, respectively. An excess of cold choline (40 mM) was aseptically added to flasks A^1, B^1 and C^1 at 1, 3 and 6 hr, respectively. These flasks were then sequentially terminated at 3, 6 and 12 after initiating the experiment. Lipids were extracted and analyzed as described in Methods. To detect choline exchange specific activities of phospholipids from flasks containing only radioactive choline were compared to those from those flasks containing excess choline that had been incubated for identical periods with labeled choline, i.e., A with A^1, B with B^1 and C with C^1, respectively.

lecular species containing the most radioactivity, i.e., diunsaturated PC, coincides with the preponderant species of PC found in milk (13) and this labeling pattern is similar to that found in other animal tissues (45). This may reflect the preponderant species of diglycerides available in ruminant mammary tissue, though available analytical data would suggest a preponderance of diglycerides with only one double bond. However the knowledge that CDP choline-diglyceride transferase is not markedly substrate specific (51) and the presence of acyl transferase(s) in mammary tissue may account for most of the radioactivity being in the PC species with two unsaturated bonds.

It is difficult to explain why more labeled choline was incorporated compared to ethanolamine, since lactating tissue secretes equal quantities of both PC and PE in milk (14,15). Possibly the entry rate of ethanolamine into the cells was much lower than that of choline, or the mammary cells had a high endogenous ethanolamine concentration compared to choline and a rapid dilution of labeled ethanolamine occurred. This is being studied. Low choline concentrations enhance the activity of the enzymes of PC synthesis in guinea pigs (52). Conceivably in lactating mammary tissue, choline is a limiting metabolite and hence used avidly when available, because the associated enzymes are very active. Choline kinase is moe active than ethanolamine kinase in bovine mammary tissue (Infante and Kinsella, unpublished, 1972). Enhanced PC synthesis is associated with intracellular membrane proliferation evoked by drugs and hormones (53-56), and possibly, in the actively secreting mammary cell, lecithin-rich intracellular membranes are

TABLE VIII

Percentage Distribution of Radioactivity in Different Phosphatidylcholine Molecules following Incubation of Bovine Mammary Cells with 1,2-^{14}C-Choline (Experiment V)

		Period of incubation, hr			
Molecular species of phosphatidylcholine	Wt %[a]	1	3	6	12
Saturated	10.2	0.4	0.1	—	0.1
Monounsaturated	44.1	31.9	35.7	20.0	14.0
Diunsaturated	27.3	48.2	51.0	50.0	48.0
Triunsaturated	10.4	15.0	8.6	27.0	20.0
Tetraunsaturated	4.4	2.5	2.8	10.0	14.0
Polyunsaturated	3.6	2.0	1.8	3.0	3.9

[a]Nutter and Privett (13).

being rapidly turned over with concomitant preferential synthesis of PC, because of their high PC content (15,57-60).

The bovine mammary gland at peak lactation secretes an average of 5% of its phospholipids into milk daily. This is comparable with turnover rates of 3.8 and 4.8% for bovine mammary protein and RNA, respectively (61). Based on concentrations in secretory tissue and in milk (14,15,19), the phospholipid classes have approximate turnover times of 29, 14 and 11 days for PC, PE and sphingomyelin, respectively. The apparent long turnover time of tissue PC compared to PE and SPH is noteworthy. Whether or not there is a relationship between the fact that significant quantities of the PE and SPH secreted in milk derived from the plasma membrane (in contrast to the PC) and their respective turnover times remains to be determined (15,19,57).

Milk phospholipids are secreted as phospholipoporteins surrounding the fat globule (1-20) and as lipopoprotein particles in milk serum (1,2,8,19,62). The latter may contain up to 30% of milk phospholipids, which are rich in PC (1,2,13,15,60). Conceivably the phospholipids of the fat globule membrane and the lipoprotein particles are secreted separately, with the latter having a much faster turnover rate. Keenan et al. (57,60) alluded to such a mechanism, suggesting that much of the water soluble components of milk are secreted in secretory vesicles derived from the endoplasmic reticulum via the membranes of the Golgi apparatus. These components, i.e., the lactose, proteins and possibly lipoprotein particles, are secreted more rapidly than milk fat globues (5,17,57,60). The secretion of milk lipoproteins via the Golgi may be analogous to the secretion of serum lipoproteins (63).

These speculations and the present results suggest that two discrete metabolic pools of PC may exist in the secreting mammary cell, i.e., a pool with a rapid turnover that is involved in intracellular metabolism, membrane replenishment and PC exchange reactions. PC molecules from this pool may behave as an interfacial surfactant for growing fat droplets (59) and may be involved in the formation of Golgi membranes and secretory vesicles, as suggested by Keenan et al. (57,60). Evidence for two pools of PC with different turnover rates has been reported in mast cells (47) and liver (44), and Gurr et al. (64) reported that during chylomicron formation in cat intestine (a process analogous to milk fat secretion) the rate of synthesis and perhaps turnover of PC is accentuated. Several workers have shown that active PC exchange occurs in cells between organelles (65-67).

Conceivably similar reactions are occurring in mammary cells. Following in vivo experiments, Patton and Keenan (19) showed that labeling of PC was much greater than PE. The PC secreted in milk lipoproteins had a 10-fold greater SA than those of the fat globule membrane and, significantly, the SA of PC in milk lipoproteins was six-fold greater than the SA of the corresponding PE. The data support previous in vitro studies showing that PC labeling exceeds that of PE (18,19,21,22), and the disparities of SA between PC and PE were of the same magnitude as observed in the present experiments.

Another pool of PC may be involved with replenishment of the plasma membrane (21) and be secreted more slowly as a component of the fat globule membrane (5,57,60). McCarthy (35) reported that mammary PL labeled with palmitic acid showed biphasic decrease in SA, i.e., a rapid phase of secretion into milk and a more prolonged phase of secretion during which SA decreased slowly. Further support for two discrete pools was recently provided by Patton et al. (68) from specific activity curves obtained following injection of 32P into lactating goat.

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REFERENCES

1. Bailie, M.J., and R.K. Morton, Biochem. J. 69:35 (1958).
2. Stewart, P., D.L. Puppione and S. Patton, Z. Zellforsch. 123:161 (1972).
3. Prentice, J.H., Dairy Sci. Abstr. 31:353 (1969).
4. Swope, F.C., and J.R. Brunner, J. Dairy Sci. 53:691 (1970).
5. Patton, S., and F.M. Fowkes, J. Theor. Biol. 15:274 (1967).
6. Patton, S., A. Durdan and R.D. McCarthy. J. Dairy Sci. 47:489 (1964).
7. Hayashi, S., D.R. Erickson and L.M. Smith, Biochemistry 4:2557 (1965).
8. Berlin, E., S. Lakshmanan, P.G. Kliman and M.J. Pallansch, Ibid. 6:1388 (1967).
9. Buckheim, W., Naturwissenschaften 12:672 (1970).
10. Keenan, T.W., D.E. Olsen and H.H. Mollenhauer, J. Dairy Sci. 54:295 (1971).
11. Keenan, T.W., D.E. Olsen and H.H. Mollenhauer, J. Dairy Sci. 54:295 (1971).
12. Huang, T.C., and A. Kuksis, Lipids 1:453 (1966).
13. Nutter, L.J., and O.S. Privett, J. Dairy Sci. 50:298 (1967).
14. Parsons, J.P., and S. Patton, J. Lipid Res. 8:696 (1967).
15. Kinsella, J.E., Lipids 7:165 (1972).

16. Morrisson, W.R., E.L. Jack and L.M. Smith, JAOCS 42:1142 (1965).
17. Kinsella, J.E., and R.D. McCarthy, Biochim. Biophys. Acta 164:530 (1968).
18. Patton, S., R.O. Mumma and R.D. McCarthy, J. Dairy Sci. 49:737 (1966).
19. Patton, S., and T. Keenan, Lipids 6:58 (1971).
20. Patton, S., L.F. Hood and J.S. Patton, J. Lipid Res. 10:260 (1969).
21. Kinsella, J.E., Biochim. Biophys. Acta 164:540 (1968).
22. Kinsella, J.E., Lipids 5:892 (1970).
23. Kinsella, J.E., J. Dairy Sci. 53:1757 (1970).
24. Kinsella, J.E., Ibid. 53:635 (1970).
25. Easter, D., S. Patton and R.D. McCarthy, Lipids 6:844 (1971).
26. Ebner, K.E., C.R. Hoover, E.C. Hageman and B.L. Larson, Exp. Cell Res. 23:373 (1961).
27. Kinsella, J.E., and R.D. McCarthy, Biochim. Biophys. Acta 164:518 (1968).
28. Folch, J., M. Lees, G.H. Sloane-Stanley, J. Biol. Chem. 226:497 (1957).
29. Rouser, G., A.N. Siakotos and S. Fleischer, Lipids 1:85 (1966).
30. Arvidson, G.A., Eur. J. Biochem. 4:478 (1968).
31. Kennedy, E.P., Fed. Proc. 20:934 (1961).
32. Annison, E.F., J.L. Linzell, S. Fazakerley and B. Nichols, Biochem. J. 102:637 (1967).
33. Dimick, P.S., R.D. McCarthy and S. Patton, Biochim. Biophys. Acta 116:159 (1966).
34. Kinsella, J.E., J. Dairy Sci. 54:1014 (1971).
35. Kinsella, J.E., Biochim. Biophys. Acta 210:28 (1970).
36. McCarthy, R.D., and S. Patton, Ibid. 70:102 (1963).
37. Bines, J.A., and R.E. Brown, J. Dairy Sci. 51:51 (1968).
38. Wright, J.D., and C. Green, Biochem. J. 123:837 (1971).
39. Bremer, J., P. Figard, D. Greenberg, Biochim. Biophys Acta 43:477 (1960).
40. Rytter, D., J.E. Miller and W.E. Cornatzer, Ibid. 152:418 (1968).
41. Artom, C., Proc. Soc. Exp. Biol. Med. 132:1025 (1969).
42. Tinoco, J., G. Sheehan, S. Hopkins and R.L. Lyman, Lipids 5:412 (1970).
43. Bjornstad, P., and J. Bremer, J. Lipid Res. 7:38 (1966).
44. Balint, J.A., D.A. Beeler, D.H. Treble and H.L. Spitzer, Ibid. 8:486 (1967).
45. Stein, O., and Y. Stein, J. Cell. Biol. 40:461 (1969).
46. Gurr, M.I., D.I. Brindley and G. Hubscher, Biochim. Biophys. Acta 98:486 (1965).
47. Pasternak, C.A., and J.J. Bergeron, Biochem. J. 119:473 (1970).
48. Kinsella, J.E., J. Dairy Sci. 52:11 (1969).
49. Isozaki, M., A. Yamamoto, T. Amako, Y. Sakai and H. Okita, Med. J., Osaka Univ. 12:285 (1962).
50. Collins, F.D., Nature 186:366 (1960).
51. Mudd, J.B., L.M. Van Golde, L.L.M. Van Deenan, Biochim. Biophys. Acta 176:547 (1969).
52. Suzuki, S., M. Kobayashi and Y. Kotake, J. Vitaminol. 6:107 (1970).
53. Morin, R.J., Experientia 26:829 (1970).
54. Young, D.L., G. Powell and W.O. McMillan, J. Lipid Res. 12:1 (1971).
55. Tata, J.R., Biochem. J. 116:617 (1970).
56. Oka, T., and Y.J. Topper, J. Biol. Chem. 246:7701 (1971).
57. Keenan, T.W., S. Patton and R. Saacke, J. Dairy Sci. 54:1349 (1971).
58. Patton, S., and L.F. Hood, in "Lactogenesis: the Initiation of Milk Secretion at Parturition," Edited by M. Reynolds and S.J. Folley, University of Pennsylvania Press, Philadelphia, Pa., 1969, p. 12.
59. Hood, L.F., and S. Patton, J. Dairy Sci., In press 1973.
60. Keenan, T.W., and C.M. Huang, Ibid. 55:1586 (1972).
61. Palmquist, D.L., and R.L. Baldwin, Ibid. 54:1505 (1971).
62. Gammack, D., and B.B. Gupta, Biochem. J. 103:729 (1967).
63. Jones, A.L., N. Ruderman and M.G. Herrera, J. Lipid Res. 8:429 (1967).
64. Gurr, M.I., D.I. Brindley and G. Hubscher, Biochim. Biophys. Acta 98:486 (1965).
65. Zilversmit, D.G., J. Lipid Res. 12:36 (1971).
66. McMurray, W.C., and R.M.C. Dawson, Biochem. J. 112:108 (1969).
67. Jungalwala, F.G., and R.M.C. Dawson, Ibid. 117:481 (1970).
68. Patton, S., R.D. McCarthy, P.E. Plantz and R. Lee, Nature 241:240 (1973).

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Fatty Acid Composition of Free Ceramides of Kidney and Cerebellum from a Patient with Farber's Disease

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ABSTRACT

The fatty acid composition of ceramides has been determined in kidney and cerebellum of a patient with Farber's disease, which is characterized by ceramidase deficiency. Farber cerebellum and kidney contained a five- and ten-fold excess, respectively, of free ceramides. The nonhydroxy fatty acid patterns of the ceramides from Farber kidney and cerebellum showed considerable similarities to those from control tissues, whereas large amounts of ceramides containing hydroxy fatty acids are found in Farber's disease tissues.

INTRODUCTION

Farber's disease (lipogranulomatosis) is a rare disorder characterized by swollen tender joints, multiple subcutaneous nodules over joints and pressure points, hoarseness and respiratory difficulty. On the basis of the clinical and pathological features, 11 cases have been reported (1).

Biochemical studies have been performed in four cases (1-6). In one of Farber's original patients a large amount of an unusual lipoglycoprotein fraction was demonstrated (2). Clausen and Rampini (3) noted a moderate excess in whole brain of phosphoglyceride and of a glycolipid fraction. Prensky et al. (4) and Moser et al. (1) studied the tissues of a severely affected patient who died when she was 9 months old and found accumulation of ceramide and ganglioside. Samuelsson and coworkers (5,6) studied the tissues of a patient who died when he was 16 years old and reported accumulation and chemical composition of ceramide in several organs.

The activities of several acid hydrolase enzymes were examined by Van Hoof and Hers (7) in a case of Farber's disease and were shown to be normal except for α-mannosidase, which was somewhat reduced. Recently we have shown that there is a deficiency of the degradative enzyme ceramidase in both kidney and cerebellum of a patient with Farber's disease (8).

In this paper we report the fatty acid composition of free ceramide isolated from kidney and cerebellum of a patient with Farber's disease, compared with ceramide from control tissues.

MATERIALS AND METHODS

Tissue

The clinical and pathological observations of the patient with Farber's disease have been published (1). The control tissues used in this study were obtained from a 3-year-old male patient who died of pneumonia (Children's Hospital, Boston, Mass., Autopsy No. A7298). The tissues, obtained at autopsy performed 6 hr after death, were placed in sealed plastic bags and stored at -60 C until they could be extracted and analyzed.

Extraction of Lipids

Pieces of frozen tissue (kidney and cerebellum white matter, 1-3 g) were excised, thawed, homogenized in a glass tissue grinder with 2 volumes of water, and lyophilized. The residue was extracted with 20 volumes chloroform-methanol 2:1 v/v following the method of Folch et al. (9). The extract was evaporated to dryness under a stream of nitrogen at 40 C.

Mild Alkali-Catalyzed Methanolysis

The total extracted lipids (0.1-0.3 g) were dissolved in 3-5 ml chloroform-0.21 N methanolic NaOH 2:1 v/v and subjected to mild alkaline methanolysis to remove glycerolipids by the method of Kishimoto and Hoshi (10).

Column and Thin Layer Chromatography

For the isolation of ceramides the residual lipids (20-30 mg) were separated by the (slightly modified) method of Hammarström (11) into four fractions by a column (1 x 10 cm) packed with 1 g Unisil (Clarkson Chemical Co. Inc., Williamsport, Pa., 100-200 mesh), equilibrated with the first solvent. Elution was carried out successively with: (a) 40 ml benzene-ethyl acetate 9:1 v/v; (b) 50 ml benzene-ethyl acetate 7:3 v/v; (c) 50 ml benzene-ethyl acetate 5:5 v/v; and (d) 40 ml chloroform-methanol 2:1 v/v. The fraction eluted with solvent (b), containing mostly nonhydroxy

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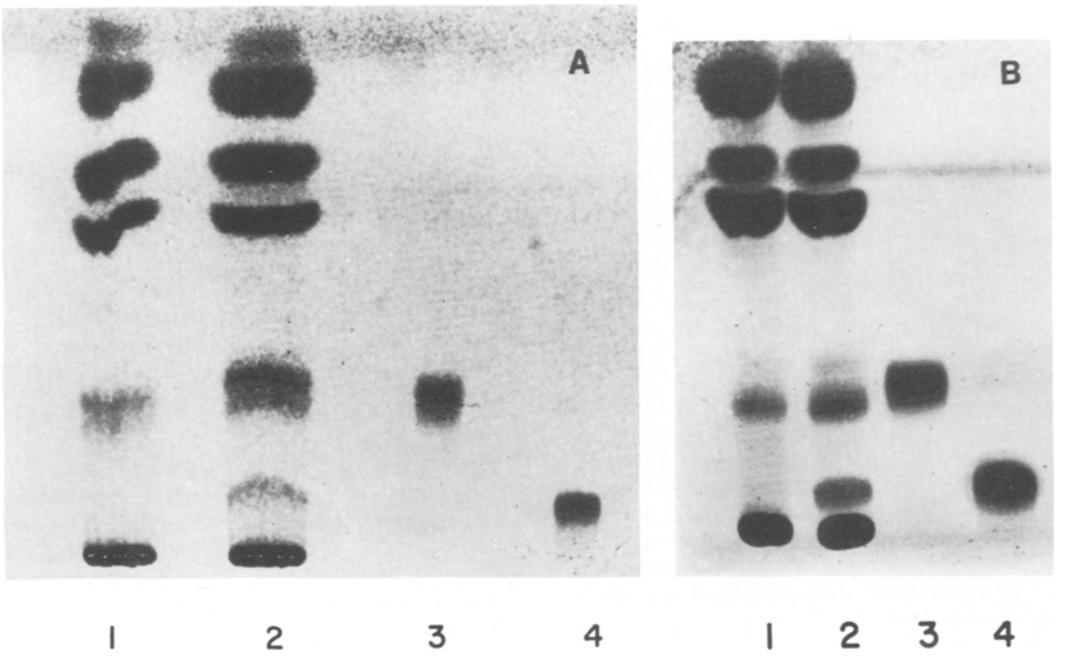

FIG. 1. Thin layer chromatogram of alkali-stable lipid fractions. Lipids of 1 g each of Farber and control kidney and cerebellum were extracted and methanolyzed as described in Materials and Methods, and the remaining sphingolipids were applied to thin layer plates and chromatographed in chloroform-methanol-acetic acid 94:1:5 v/v/v; lipids were detected by charring after spraying with 95% H_2SO_4. A, Kidney sphingolipids; B, cerebellum sphingolipids. 1, Control tissue; 2, Farber tissue; 3, standard NFA-ceramides; 4, standard HFA-ceramides.

fatty acid (NFA) ceramide, and that eluted with solvent (c), containing mostly hydroxy fatty acid (HFA) ceramide, were subjected separately to preparative thin layer chromatography (TLC) on Silica Gel G plates (20 x 20 cm, thickness 0.25 mm, Analtech Inc., Newark, Del.), using chloroform-methanol-acetic acid 94:1:5 v/v/v as developing solvent. Ceramides containing NFA had greater mobility than those containing HFA and were identified by comparison of their R_f values with those of authentic ceramides from bovine cerebrosides (Supelco, Inc., Bellefonte, Pa., and Sigma Chemical Co., St. Louis, Mo.). Exposure to iodine vapor was employed to visualize lipids on the developed TLC plates. All gel portions containing NFA-ceramide from a given organ were scraped from the plates and packed into one column (1 x 10 cm); gel containing the HFA-ceramides was treated similarly. The ceramides were recovered by elution with 30 ml chloroform-methanol 2:1 v/v.

Quantitative Analysis of Ceramides

Ceramide was hydrolyzed by the method of Gaver and Sweeley (12), and the sphingosine content (= ceramide content) was determined by the method of Lauter and Trams (13).

Preparation of Fatty Acid Methyl Esters

Methanolysis of the ceramide (0.1-0.5 mg) was carried out in methanolic HCl. Reaction mixtures containing 0.5 ml 0.5 N HCl in methanol were heated at 100 C for 3 hr in sealed tubes. After cooling, methyl esters of fatty acids were extracted three times with 0.7 ml volumes of *n*-hexane. The *n*-hexane was evaporated, and the fatty acid methyl esters obtained were purified by Unisil (equilibrated with the first solvent) column chromatography with: (a) hexane-benzene 8:2 v/v; (b) hexane-benzene 6:4 v/v; and (c) benzene as elution solvents. The fraction eluted with solvent (b) contained only NFA methyl esters and that from solvent (c) contained only HFA methyl esters. As standards, methyl palmitate and methyl α-hydroxy palmitate were used to determine the behavior of fatty acid methyl esters on this Unisil column, followed by TLC on Silica Gel G using hexane-ether 8:2 v/v as solvent (10).

Gas Liquid Chromatographic Analysis

Gas liquid chromatographic analysis was performed on a Hewlett-Packard Model 7620A gas chromatograph equipped with a hydrogen flame ionization detector. The analytical col-

TABLE I

Concentration of Free Ceramide Isolated from Kidney and Cerebellum of Control and Farber's Disease[a]

Ceramides	Kidney[b]		Cerebellum[b]	
	Control	Farber	Control	Farber
NFA-Ceramide[c]	30	159	63	187
HFA-Ceramide[c]	—	121	—	124
Total	30	280	63	311

[a]Ceramides were prepared and analyzed for sphingosine content as described in Materials and Methods.

[b]Data are expressed as micrograms ceramide per gram wet tissue.

[c]NFA, nonhydroxy fatty acid; HFA, hydroxy fatty acid.

umn (2 mm x 2 M) was packed with 3% OV-1 coated on 80-100 mesh Chromosorb W (AW-DMCS) (Applied Science Labs. Inc., State College, Pa.). The carrier gas was helium.

For analysis of the methyl esters of NFA and HFA, the column temperature was programed to increase 1 C/min, starting at 170 and 190 C, respectively. The methyl esters of HFA were analyzed as their trimethylsilylated (TMSi) derivatives as follows (10). The methyl esters of HFA were heated at 60-70 C for 5-6 min with the TMSi reagents. After reaction, 5 ml chloroform and 1 ml water were added to the reaction mixture and the TMSi derivatives were extracted with chloroform. The chloroform layer was washed several times with 1 ml water and evaporated under a stream of nitrogen. The various peaks on the gas chromatogram were identified by comparison with a calibration curve, using the methyl ester mixtures of NFA (KD and KF, Applied Science Labs.), or with the methyl esters of HFA of bovine cerebroside (Supelco, Inc.).

RESULTS AND DISCUSSION

The cerebellum and kidney of the patient with Farber's disease contained a five- and ten-fold excess, respectively, of total free ceramide when compared with control tissues (Table I); the ceramide containing HFA is seen to amount to ca. 40% of the total ceramide accumulated in Farber tissues, whereas HFA was not detectable in control tissues. This is also clearly seen in Figure 1. An excess of free ceramides above control values in Farber visceral organs including liver (60-fold the control values), lung (30-fold) and kidney (10-fold), and in brain white matter (5-fold) was previously demonstrated in our laboratory (1). Also Samulesson et al. (6) have determined the ceramide concentration and the fatty acid composition of the ceramide in Farber kidney, liver, lung, spleen and brain white and grey matter; however, unlike the case reported here, ceramide levels in liver and brain white matter (1) were normal. Samuelsson et al. reported only trace amounts of HFA-ceramide in Farber brain, but observed the same striking elevation in kidney HFA-ceramide as shown here.

The presence of HFA noted here is an exception to the repeated observation that normally occurring ceramide contains only NFA. Rouser and Yamamoto (14) have reported that ceramide containing HFA was not detectable upon TLC of human whole brain lipid extract. In addition, both Samuelsson (15) and Krivit and Hammarström (16) have remarked on the absence of HFA in ceramide of human plasma and platelets, respectively.

The fatty acid composition of ceramide from control and Farber kidney and cerebellum is shown in Tables II and III. In kidney, the distribution of NFA in ceramide from Farber's disease was similar to that found in control ceramide except for $C_{18:1}$ and $C_{24:1}$ acids, whose proportions in Farber kidney seem to be reversed from those in control kidney. The ceramide from Farber and control cerebellum showed great similarity in NFA composition, consisting mainly of $C_{18:0}$ acid (Farber, 56.2% of total NFA; control, 60.3%). In both Farber tissues, kidney and cerebellum, HFA was constituted mostly by the longer chain fatty acids ($C_{22h:0}$ and above), predominantly $C_{24h:0}$. HFA of the cerebellum contained only trace amounts of $C_{24h:1}$ acid, but that from kidney contained $C_{24h:1}$ acid amounting to 10.4% of total HFA. Samuelsson and coworkers (6) reported finding high percentages of $C_{18:0}$ and $C_{24:1}$ in both control and Farber brain white and grey matter. They found the major NFA constituent of Farber and control kidney ceramide to be $C_{24:1}$; the major HFA of Farber kidney ceramide was $C_{24h:0}$, similar to the results reported here. The HFA composition of the ceramide of liver of the patient reported here (1) is similar to the HFA composition of ceramide of kidney and cerebellum, containing

TABLE II

Nonhydroxy Fatty Acid Composition of Free Ceramide from Kidney and Cerebellum of Control and Farber's Disease[a]

Fatty acid	Kidney[b]		Cerebellum[b]	
	Control	Farber	Control	Farber
16:0	20.2	15.7	2.5	1.3
17:1	—	—	0.7	0.7
17:0	4.3	0.6	tr	tr
18:1	10.2	1.0	5.5	2.3
18:0	14.0	7.8	60.3	56.2
19:1	—	—	2.2	4.1
20:1	tr[c]	tr	tr	tr
20:0	4.4	4.7	7.3	3.9
21:0	tr	0.1	0.6	tr
22:1	tr	0.6	1.1	1.1
22:0	12.8	17.7	1.8	2.1
23:1	—	—	tr	tr
23:0	4.4	3.5	0.6	0.8
24:1	4.1	19.4	7.9	6.7
24:0	25.1	28.0	2.6	6.4
25:1	tr	tr	2.6	7.6
26:1	—	—	1.9	2.9

[a]Methyl esters of ceramide nonhydroxy fatty acids were prepared and analyzed by gas liquid chromatography as described in Materials and Methods.

[b]Data are expressed as per cent of total nonhydroxy fatty acid in ceramide of the designated organ.

[c]tr = trace.

a high proportion of $C_{22h:0}$ (20.7%) and $C_{24h:0}$ (27.6%).

It has already been noted that, in the present Farber's disease case, free ceramides were accumulated in nearly all the tissues examined (1), whereas in the case reported by Samuelsson and Zetterström (5) ceramide excess was found in the kidney and in the subcutaneous nodule, but not in the liver, brain or lung. Hydroxy fatty acids are not demonstrable in control tissues, nor in those Farber's disease tissues in which total ceramide levels are normal. In contrast, those Farber's disease tissues in which ceramide levels are increased do contain substantial amounts of hydroxy fatty acids.

TABLE III

Hydroxy Fatty Acid Composition of Free Ceramide from Kidney and Cerebellum of Farber's Disease[a]

Fatty acid	Kidney[b]	Cerebellum[b]
14*h*:0	4.2	—
16*h*:1	1.8	—
16*h*:0	5.6	0.8
17*h*:0	—	3.8
18*h*:0	3.1	4.7
20*h*:0	5.4	4.2
21*h*:0	tr[c]	0.6
22*h*:0	16.6	11.8
23*h*:0	11.2	7.0
24*h*:1	10.4	tr
24*h*:0	41.6	55.8
25*h*:0	—	3.3
26*h*:1	—	2.6
26*h*:0	—	5.3

[a]Trimethylsilylated derivatives of ceramide hydroxy fatty acid methyl esters were prepared and analyzed as described in Materials and Methods.

[b]Data are expressed as per cent of total hydroxy fatty acid.

[c]tr = trace.

We have observed that normal human kidney and cerebellum both contain ceramidase and ceramide synthetase activities with optimal pH of 4, and that normal cerebellum has in addition ceramidase and ceramide synthetase activities at pH 9, which, in contrast to the pH 4 enzymes, remain active in Farber cerebellum (17).

At this time the most plausible explanation for the ceramide and sphingolipid accumulation in Farber's disease is the deficient acid ceramidase activity. The fact that patients with Farber's disease have normal or increased levels of sphingolipids (1) indicates that sphingolipid synthesis, and hence ceramide synthesis, can proceed in the absence of that ceramide synthetase that has a pH optimum of 4.0. The observation that cerebellum pH 9 ceramidase and ceramide synthetase activities appear normal in this disease, and the finding that normally the ratio of pH 4 to pH 9 ceramidase and ceramide synthetase activities, and the specific acitivities of these enzymes, are not the same in different organs (17), may help account for the fact that the extent of ceramide accumulation is not the same in the various tissues, as well as for the differences in ceram-

ide levels noted in the two cases so far examined.

It is not possible to say at this point from which spingolipids the accumulated ceramide might be derived, although the strikingly large amount of HFA-ceramide found in organs where ceramide accumulates suggests that cerebroside is one of the main precursors. It is known that ceramide may be derived from cerebrosides (18) and that cerebrosides contain large amounts of HFA. Furthermore the HFA pattern in the accumulated Farber ceramide resembles that of normal brain and kidney cerebroside. In particular, human brain cerebroside contains considerable amounts of $C_{18:0}$ and $C_{24h:0}$ acids (19-21), while human kidney cerebroside contains relatively large amounts of $C_{22:0}$, $C_{24:1}$ and $C_{24:0}$ NFA, and $C_{22h:0}$, $C_{23h:0}$, $C_{24h:1}$ and $C_{24h:0}$ HFA (22). We presume that the HFA-ceramide formed from cerebrosides and other sources in normal tissues is degraded or reutilized rapidly enough that its presence is barely demonstrable.

The fatty acid pattern of NFA-ceramide seen here, while bearing a resemblance to that of cerebroside, as mentioned above, is not distinctive enough to permit extensive speculation about its origin. Human brain sphingomyelin has been shown (19) to contain very large amounts of $C_{18:0}$, similar to the results reported here for control and Farber cerebellum ceramide, but no detectable HFA, unlike our findings for Farber cerebellum ceramide. Human brain gangliosides, while containing considerable amounts of $C_{18:0}$ NFA, also contain high proportions of C_{20}-sphingosine (23); we have observed that this long chain base is not detectable (17) in ceramide of the Farber case discussed here, similar to the findings of Samuelsson and Zetterström (5). This suggests that only a relatively small proportion of the free ceramide accumulated in Farber's disease is derived from ganglioside. It should also be noted that Klenk and Huang (24) have observed that in human grey matter fractions containing ceramide and sphingomyelin rich in $C_{18:0}$, significant amounts of C_{20}-sphingosine are present, whereas white matter ceramide and sphingomyelin, also rich in $C_{18:0}$ acid, contain only trace amounts of C_{20}-sphingosine. It will be recalled that the type of brain tissue examined in the present work was predominently white matter.

We consider it likely that the fatty acid composition of Farber ceramide would reflect the relative activities and substrate specificities of the ceramide synthetic and degradative enzymes that are still active in Farber's disease tissues. We are now examining the substrate specificities of the ceramidase and ceramide synthetase that are active in at least some tissues in Farber's disease, e.g., pH 9, cerebellum, referred to above. Also the synthetases so far discussed utilize free fatty acids as substrates; fatty acyl CoA derivatives also serve as fatty acid donors in the biosynthesis of ceramide (25), and it may be that such activities are present in Farber, as well as in control, tissues. This possibility is also under investigation.

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REFERENCES

1. Moser, H.W., A.L. Prensky, H.J. Wolfe and N.P. Rosman, Amer. J. Med. 47:869 (1969).
2. Farber, S., J. Cohen and L.L. Uzman, J. Mt. Sinai Hosp. 24:816 (1957).
3. Clausen, J., and S. Rampini, Acta Neurol. Scand. 46:313 (1970).
4. Prensky, A.L., G. Ferreira, S. Carr and H.W. Moser, Proc. Soc. Exp. Biol. Med. 126:725 (1967).
5. Samuelsson, K., and R. Zetterström, Scand. J. Clin. Lab. Invest. 27:393 (1971).
6. Samuelsson, K., R. Zetterström and B.I. Ivemark, "Sphingolipids, Sphingolipidoses and Allied Disorders," Edited by B.W. Volk and S.M. Aronson, Plenum Publishing Co., New York, 1972, p. 533.
7. Van Hoof, F., and H.G. Hers, Eurp. J. Biochem. 7:34 (1968).
8. Sugita, M., J.T. Dulaney and H.W. Moser, Science 178:1100 (1972).
9. Folch, J., M. Lees and G.H. Sloane-Stanley, J. Biol. Chem. 226:497 (1957).
10. Kishimoto, Y., and M. Hoshi, "Methods of Neurochemistry," Vol. 3, Edited by R. Fried, Marcel Dekker Inc., New York, 1972, p. 75.
11. Hammarström, S., J. Lipid Res. 12:760 (1971).
12. Gaver, R.C., and C.C. Sweeley, JAOCS 42:294 (1965).
13. Lauter, C.J., and E.G. Trams, J. Lipid Res. 3:136 (1962).
14. Rouser, G., and A. Yamamoto, Lipids 7:561 (1972).
15. Samuelsson, K., Scand. J. Clin. Lab. Invest. 27:371 (1971).
16. Krivit, W., and S. Hammarström, J. Lipid Res. 13:525 (1972).
17. Sugita, M., P. Connolly, J.T. Dulaney and H.W. Moser, "Fourth International Society for Neurochemistry (Abstract)," Tokyo, Japan, 1973.
18. Morell, P., and P. Braun, J. Lipid Res. 13:293 (1972).
19. O'Brien, J.S., and E.L. Sampson, Ibid. 6:545 (1965).
20. Menkes, J.H., M. Philippart and M.C. Concone, Ibid. 7:479 (1966).
21. Svennerholm, L., and S. Stallberg-Stenhagen, Ibid. 8:215 (1968).

22. Martensson, E., Biochim. Biophys. Acta 116:521 (1968).
23. Rosenberg, A., and N. Stern, J. Lipid Res. 7:122 (1966).
24. Klenk, E., and R.T.C. Huang, Hoppe-Seylers Z. Physiol. Chemie 350:373 (1969).
25. Sribney, M., Biochim. Biophys. Acta 125:542 (1966).

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Cyclopropenoic Acids of *Pavonia sepium* Seed Oil[1]

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ABSTRACT

The seed oil of *Pavonia sepium* (Malvaceae) contains sterculic (7%) and malvalic (4%) acids. Countercurrent distribution in a hexane-acetonitrile system concentrated and resolved the homologus cyclopropenoid methyl esters. Reductive ozonolysis of selected countercurrent distribution fractions gave β-diketoesters, which were identified by gas chromatography-mass spectrometry as derivatives of sterculic and malvalic acids. *Pavonia sepium* is the first known species in the plant family Malvaceae whose seed oil contains more sterculic than malvalic acid.

Pavonia sepium St. Hil. (Malvaceae) is a member of a plant family whose seed oils contain cyclopropenoic and epoxy acids (1). Titration of its seed oil with HBr (2) indicated that these acids are present. This paper describes the characterization of the cyclopropenoic acids of this oil.

Seed oils were obtained by extraction of the coarsely ground seeds with petroleum ether (bp 35-60 C) followed by evaporation of the solvent. Mixed methyl esters were obtained by NaOMe/MeOH transesterification. The two-temperature HBr titration showed a cyclopropenoic ester content of 13.9% (as methyl sterculate) and an epoxy ester content of 3.7% (as methyl epoxyoleate). Countercurrent distribution (CCD) (3) of the esters in a hexane-acetonitrile system, followed by evaporating selected fractions from 700 transfers, gave a weight curve (Fig. 1) with peaks at transfers 258, 298 and 370, representing fractions rich in stearate, oleate and linoleate, respectively.

Quantitative IR analysis in CS_2 solution was run on nine fractions from CCD. The absorbance (A) at 9.9 μm, which is characteristic of cyclopropenes (4), was measured using the background absorbance correction technique (5). By comparing the absorbances with a straight line calibration curve obtained by plotting A vs. milligrams methyl sterculate in several dilutions of *Sterculia foetida* methyl esters (71.3% methyl sterculate by HBr titration) and applying the values obtained to the CCD weight curve, a cyclopropenoic methyl ester weight curve (broken line in Fig. 1) was derived. Other workers have determined sterculic acid by IR in CS_2 solution (6) and methyl sterculate in CCl_4 solution (5). When CCl_4 is used in the IR determination of cyclopropenes, a fairly concentrated solution is required because of the absorbance of this solvent in the region of 9.9 μm. In contrast, CS_2 is transparent in this portion of the spectrum. CS_2 reacts with cyclopropenes on heating or long standing (7), but we found that results were reproducible if the solutions are kept at ambient temperature and are used immediately after they are prepared.

Three maxima in the cyclopropene weight curve were revealed and samples under each of these peaks (areas 1, 2 and 3) were withdrawn for ozonolyses carried out in ethyl acetate (100 mg/10 ml) at 0 C. Palladium-on-carbon catalyst was then added and the solutions were hydrogenated at 27 C and atmospheric pressure. Ozonolysis products were analyzed on gas liquid chromatography (GLC) and by thin layer chromatography (TLC). Diketones resulting from the cleavage reaction were concentrated for further analysis by preparative TLC. Analytical TLC was carried out on glass plates coated with Silica Gel G, which were developed with hexane-ethyl ether-acetic acid 80:20:2 and visualized by charring with sulfuric acid-chromic acid. Diketo esters were visualized with 3% alcoholic $FeCl_3$; a deep red spot is charac-

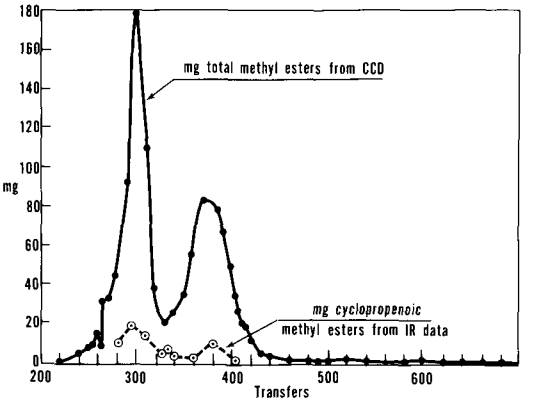

FIG. 1. Weight distribution from countercurrent distribution (CCD) of mixed methyl esters from *Pavonia sepium* seed oil.

[1]Presented at the AOCS-ISF World Congress, Chicago, October 1970.

[2]ARS, USDA.

teristic of 1,3-diketo compounds (8). Preparative TLC was conducted with 1 mm layers of Silica Gel G. Ozonolysis products were analyzed by GLC in two ways: (a) on a Packard Model 7401 gas chromatograph having a 4 ft x 1/8 in. glass column packed with 3% Apiezon L (ApL) on Gas Chrom Q and equipped with a flame ionization detector, column temperature 200 C; and (b) on a Burrell Kromotog Model KD chromatograph equipped with a 2 ft x 3/8 in. stainless steel column packed with 5% LAC-2-R446 (Resoflex) on Chromasorb W-AWDMCS. The apparatus had a flame ionization detector and column temperature was 180 C. Equivalent chain length (ECL) values were determined by the method of Miwa et al. (9,10). Because of the high ECL values (and consequently long retention times), Resoflex columns were not used for quantitative analysis of the reduced ozonides. However the 2 ft Resoflex column described above was used to determined ECL's. Reduced ozonide fractions giving ECL values on ApL at 19.4, 20.4 and 21.4 had ECL's of 29.5, 30.5 and 31.5, respectively, on the Resoflex column. These values are those that would be expected for a series of 1,3-diketo esters having 17, 18 and 19 carbons, respectively (10). The diketo ester concentrates were subsequently analyzed by gas chromatography-mass spectrometry (GC-MS) with instrumentation and operating conditions as described by Kleiman and Spencer (11).

GLC (ApL) of the reduced ozonide mixture from area 1 (transfers 288-309) showed 79% of a component with ECL 16.0. Other significant peaks were at ECL 19.2 (0.03%), 20.4 (1.0%) and 21.3 (2.5%). Preparative TLC gave a yellow band at R_f 0.4, which on analytical TLC gave one spot that corresponded to a red spot observed with the $FeCl_3$ reagent. GLC analysis (ApL) indicated components at ECL 19.2 (1%), 20.4 (24%) and 21.4 (63%). GC-MS gave a spectrum for the ECL 20.4 peak with a molecular ion at m/e 326. This spectrum was identical to that of methyl 8,10-diketo-octadecanoate (from malvalic acid) as reported by Hooper and Law (12). The ECL 21.4 peak with a molecular ion at m/e 340 gave a spectrum identical to that of methyl 9,11-diketononadecanoate (from sterculic acid) (12).

GLC of the reduced ozonides from area 2 (transfers 322-347) showed a mixture having ca. 21% at ECL 16.0 and peaks at ECL 20.2 (24.6%) and 21.3 (1.9%). By preparative TLC, the diketones were concentrated in a yellow band whose GLC analysis (ApL) showed components with the following ECL values: 19.4 (0.14%), 20.3 (89.2%) and 21.3 (7.7%). The mass spectrum of the ECL 20.3 peak showed a molecular ion at m/e 326 and was identical to that of methyl 8,10-diketo-octadecanoate, while the ECL 21.3 peak again gave the spectrum (molecular ion, m/e 340) identical to that of 9,11-diketononadecanoate. GLC (ApL) of the mixed reduced ozonides area 3 (transfer 360-381) showed only a small amount of material in the ECL region of interest with 0.9% at 19.4 and 0.3% at 20.4. Preparative TLC gave a band that, when eluted and analyzed by GLC (ApL), included components with the following ECL values: 19.4 (9%), 20.4 (0.7%) and 21.6 (0.9%). This fraction had the greatest content of material with ECL 19.4 obtained from any of the areas. GC-MS of the ECL 20.4 and 21.6 peaks gave the expected spectra for the diketo methyl esters from malvalic and sterculic acids, respectively, but the mass spectrum for the ECL 19.4 peak was inconclusive.

It is noteworthy that CCD was successful in separating methyl esters of cyclopropenoic acids differing in chain length by only one methylene group. Usually such separations have been facilitated by a difference of two methylene groups. The peaks on the cyclopropene weight curve (Fig. 1) represent fractions rich in sterculate (transfer 294), malvalate (transfer 334) and unknown (transfer 380), respectively. Taken at face value, these results indicate that *Pavonia sepium* seed oil contains 7% sterculate, 4% malvalate and 3% of an unknown cyclopropene having 17 carbons. However we were unable to obtain confirmation for the structure of the apparent C_{17}-diketoester by GC-MS. This difficulty may have been due to the relatively low concentration of the component with ECL 19.4 in the sample that was analyzed. Thus, although we found indications of a C_{17}-cyclopropenoic acid in *Pavonia* seed oil, we did not establish this definitely.

Over a period of years, other investigators have found in various species indications of elusive cyclopropenes with chain lengths shorter than malvalic acid. Raju and Reiser (13) reported evidence for a cyclopropenoic acid with a GLC retention time shorter than malvalic in *Althaea rosea* (Cav.) seed oil. Johnson et al. (14 and references cited therein) hinted at the occurrence of C_{17}-cyclopropenes in the fruits of certain *Malva* species. More recently, Ackman and Hooper (15) encountered an unusual component in *Euphoria longans* seed oil which they regarded as possibly being a C_{12}-cyclopropenoid acid.

P. sepium seed oil is unusual in being the first of the family *Malvaceae* in which the content of sterculic acid was observed to be greater than that of malvalic acid. This trait has

been found only in one example in the Sterculiaceae (*S. foetida*) (10,16) and one in the Bombacaceae (*Bombacopsis glabra*) (17).

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REFERENCES

1. Smith, C.R., Jr., in "Progress in the Chemistry of Fats and Other Lipids," Vol. XI, Part 1, Edited by R.T. Holman, Pergamon Press, 1970, p. 137.
2. Harris, J.A., F.C. Magne and E.L. Skau, JAOCS 40:718 (1963).
3. Scholfield, C.R., J. Nowakowska and H.J. Dutton, Ibid. 37:27 (1960).
4. Faure, P.K., and J.C. Smith, J. Chem. Soc. 1956:1818.
5. Bailey, A.V., G.J. Boudreaux and E.L. Skau, JAOCS 42:637 (1965).
6. Varma, J.P., S. Dasgupta, B. Nath and J.S. Aggarwal, Ibid. 34:452 (1957).
7. Faure, P.K., Nature (London) 178:372 (1956).
8. Nunn, J.R., J. Chem. Soc. 1952:313.
9. Miwa, T.K., K.L. Mikolajczak, F.R. Earle and I.A. Wolff, Anal. Chem. 32:1739 (1960).
10. Wilson, T.L., C.R. Smith, Jr., and K.L. Mikolajczak, JAOCS 38:696 (1961).
11. Kleiman, R., and G.F. Spencer, Ibid. 50:31 (1973).
12. Hooper, N.K., and J.H. Law, J. Lipid Res. 9:270 (1968).
13. Raju, P.K., and R. Reiser, Lipids 1:10 (1966).
14. Johnson, A.R., J.A. Pearson, F.S. Shenstone, A.C. Fogerty and J. Giovanelli, Ibid. 2:308 (1967).
15. Ackman, R.G., and S.N. Hooper, JAOCS 47:525 (1970).
16. Shenstone, F.S., and J.R. Vickery, Nature (London) 190:168 (1961).
17. Cornelius, J.A., T.W. Hammonds and G.G. Shone, J. Sci. Food Agr. 16:170 (1965).

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Incorporation of [1-^{14}C]-Oleic Acid into Neutral Glycerides and Phosphoglycerides of Mouse Brain

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ABSTRACT

The incorporation of [1-^{14}C]-oleic acid into the neutral glycerides and phosphoglycerides of adult mouse brain was examined between 1 and 80 min after intracerebral injection. Radioactivity of the free oleic acid in brain decreased rapidly with a half-life of ca. 5 min. The specific radioactivity of the phosphatidic acids was highest at 1 min after injection. This was followed by the diacylglycerols and triacylglycerols which attained a maximum specific radioactivity at 3 and 20 min after injection, respectively. Specific radioactivities of the neutral glycerides were higher than the phosphoglycerides. A larger proportion of the radioactivity in the diacylglycerols was transferred to the phosphoglycerides than to the triacylglycerols. Among the phosphoglycerides, radioactivity was actively incorporated into the inositol phosphoglycerides. The specific radioactivity of the inositol phosphoglycerides was higher than the diacyl *sn*-glycero-3-phosphorylcholines, and the kinetics of incorporation of radioactivity into these lipids was also different. A water soluble material was found which showed maximum specific radioactivity at 6-10 min after injection. The properties of this water soluble material suggested that it may be an intermediate involved in the acyl group metabolism of phosphoglycerides in brain.

INTRODUCTION

The study of brain fatty acid metabolism by intracerebral injection of labeled precursors (1-5) is advantageous over oral, intraperitoneal or intravenous injections (6-8) because a much higher proportion of the administered dose can be incorporated and metabolized directly by the brain tissue. On the other hand, administration of labeled precursors by these other techniques as well as by carotid injection (9) has revealed that the fatty acids can be directly incorporated into the brain tissue without prior oxidation to acetate.

Although brain lipids may be labeled in vivo by many types of precursors, the information gained is influenced by the choice of precursors. The use of choline (10,11) or ethanolamine (12,13) as precursors is restricted to comparisons of subclasses of the respective phosphoglycerides. Acetate and glucose (14,15) are incorporated not only into all lipid classes but also into all moieties of the lipid molecules. Exhaustive analyses are required in order to interpret the results. The use of [^{32}P]-phosphate (11,16) is restricted to comparisons of phospholipids. Previous studies with [U-^{14}C]-palmitic acid as precursor have indicated that the fatty acid is metabolized efficiently in brain with a half-life of less than 5 min (2), and is an excellent precursor for the study of brain lipid metabolism (1,2) and turnover (17). However palmitoyl groups are associated more closely with the choline phosphoglycerides than the ethanolamine and serine phosphoglycerides (18). Since oleic acid is a major component of the brain phosphoglycerides, we have examined the uptake and incorporation of [^{14}C]-oleic acid in the adult mouse brain between 1 and 80 min after intracerebral injection of the labeled precursor.

MATERIALS AND METHODS

Female C57BL/10J mice, 6 months of age, were injected intracerebrally with [1-^{14}C]-oleic acid (Applied Science Labs., Inc., State College, Pa.; specific radioactivity 53 mCi/mmol) complexed with bovine serum albumin (1). The procedure for intracerebral injection of labeled precursor into the mouse brain without anesthesia has been described (13). Each animal received 15 μl of the emulsion containing ca. 0.5 μc of the [^{14}C]-oleic acid. At 1, 3, 6, 10, 20, 40 and 80 min after injection, three mice were killed and the brains were individually homogenized in 20 volumes chloroform-methanol 2:1 v/v. Then 0.2 volumes water was added to the lipid extract (19), and portions of the upper phase and the lower phase were taken for measurement of radioactivity. The lipid extract from each brain was dissolved in 10 ml chloroform and stored at 4 C until further analysis.

The procedure for separation of lipids into the major classes by Unisil silicic acid column chromatography has been described previously (1). The less polar lipids were separated on thin layer chromatographic (TLC) plates developed

TABLE I

Content of Radioactivity in Organic and Aqueous Phases of Brain Lipid Extract after Intracerebral Injection of [1-^{14}C]-Oleic Acid[a]

Min after injection	dpm x 10^{-3}/brain		Aqueous/organic, %
	Organic phase	Aqueous phase	
1	120 ± 2	6.0 ± 0.7	5.0
3	153 ± 16	10.4 ± 1.7	6.8
6	155 ± 50	16.4 ± 3.5	10.6
10	103 ± 9	13.6 ± 1.5	13.2
20	102 ± 12	8.5 ± 1.8	8.3
40	143 ± 7	6.4 ± 1.9	4.5
80	131 ± 10	3.0 ± 1.1	2.3

[a]Results are mean ± SEM from three brains. Each brain was dispersed in 20 volumes chloroform-methanol 2:1 v/v and 0.2 volumes of water was added. Portions of aqueous and and organic phases were taken for measurement of radioactivity.

twice with hexane-diethyl ether 70:30 v/v. Individual phospholipids were separated by the separation-HCl reaction-separation TLC procedure developed with chloroform-methanol-15N NH_4OH 65:25:4 v/v for the first dimension, and chloroform-methanol-acetone-acetic acid-water 75:15:30:15:7.5 v/v for the second dimension (20). Lipid spots were visualized either by exposing the TLC plates to iodine vapor or by spraying the plates with 2′,7′-dichlorofluorescein reagent. The phosphorus content of individual phospholipids was determined according to the procedure of Gottfried (21). For determination of radioactivity, each lipid spot was scraped into a vial containing 10 ml XDC scintillation fluid (13).

The acyl group composition of individual glycerides was determined after TLC separations of the neutral glycerides and phosphoglycerides obtained after column fractionation. The acyl groups were converted to their methyl esters by alkaline methanolysis and analyzed by gas liquid chromatography (20). A known quantity of heptadecanoic acid ($C_{17:0}$) was added to each sample as internal standard. The oleoyl group content of individual glycerides was obtained by comparing the peak area of the internal standard with the oleoyl group from individual glyceride molecules.

The methyl esters from the phospholipids were separated according to unsaturation by TLC plates impregnated with $AgNO_3$. A solvent system of hexane-diethyl ether 80:20 v/v was used for the separation. Individual spots containing the methyl esters were scraped for measurement of radioactivity.

RESULTS

Although the labeled precursor used for the injection is a lipid material that is completely soluble in the organic solvent, a small amount of the radioactivity was found in the aqueous upper phase during lipid extraction and phase partition (Table I). Indeed, the radioactivity in the aqueous phase increased with time and

TABLE II

Distribution of Radioactivity among Major Lipid Classes after Intracerebral Injection of [1-^{14}C]-Oleic Acid[a]

Min after injection	Per cent			
	Free fatty acids[b]	Neutral glycerides[b]	Galactolipids	Phosphoglycerides
1	79.0	7.1	2.5	11.4
3	56.7	20.5	2.1	20.8
6	38.5	22.6	1.3	37.6
10	13.6	28.2	2.3	55.9
20	5.9	22.7	1.9	69.4
40	2.7	18.9	2.0	77.0
80	3.3	11.4	1.0	84.3

[a]Lipid extract from each mouse brain was placed on a Unisil silicic acid column. Procedure for elution of the lipids has been described (1). Each value is the mean percentage from three brains.

[b]Radioactivities of free fatty acids and neutral glycerides were obtained after thin layer chromatographic separation of less polar lipid fraction.

TABLE III

Percentage Distribution of Radioactivity among Phosphoglycerides and Neutral Glycerides of Mouse Brain Lipids[a]

Min after injection	Per cent[b]							
	DG	TG	PA	IPG	SPG	Diacyl GPC	Diacyl GPE	Alkenyl acyl GPE[c]
1	31.1	7.0	13.1	9.5	7.5	21.0	9.5	1.3
3	38.2	10.5	3.8	12.8	3.4	19.3	10.8	1.2
6	16.3	20.8	1.6	12.9	1.1	31.6	14.4	1.2
10	13.7	19.1	1.4	14.5	2.1	32.5	17.4	1.3
20	6.3	18.1	---	12.6	2.2	38.8	20.4	1.6
40	2.8	16.2	1.1	12.6	2.0	44.9	18.8	1.5
80	2.6	8.8	1.1	13.8	2.9	46.1	22.5	1.3

[a]Procedure for separation of phosphoglycerides and neutral glycerides by thin layer chromatography is described in the text. Radioactivity of free fatty acids is not included. Each value is mean percentage from three brains.

[b]Abbreviations: DG, diacylglycerols; TG, triacylglycerols; PA, phosphatidic acids; IPG, inositol phosphoglycerides; SPG, serine phosphoglycerides; GPC, *sn*-glycero-3-phosphorylcholine; GPE, *sn*-glycero-3-phosphorylethanolamine.

[c]Radioactivity of acyl group of alkenyl acyl GPE.

reached a maximum 6 min after injection. At 10 min after injection, the proportion of radioactivity in the aqueous phase accounted for 13% of the total radioactivity in brain. Further examination of this radioactive material indicated that it was dialyzable and was rather resistant to either acid or alkaline methanolysis.

Approximately 14% of the labeled carbon from the injected oleate was recovered in the mouse brain after intracerebral injection. The half-life for this fatty acid in brain was less than 5 min (Table II). In spite of the rapid decline in radioactivity of the labeled precursor, the total radioactivity in brain remained nearly constant. The rapid decrease in radioactivity of the free fatty acid was accompanied by an increase in the radioactivity of the neutral glycerides and phosphoglycerides (Table II). At 80 min after injection, 84% of the radioactivity of the brain lipids was in the phospholipids and only 3% of the radioactivity was still in the free fatty acids. The radioactivity of the neutral glycerides, however, reached a maximum (28%) at 10 min and then decreased to 11% at 80 min.

Separation of the acyl groups from phosphoglycerides by argentation TLC indicated that over 90% of the radioactivity was still in the monoene band. At all times radioactivity of the galactolipids was very low, and therefore they were not subjected to further analysis.

The percentage distribution of radioactivity among the individual neutral glycerides and phosphoglycerides is shown in Table III. At 1 min after injection the level of radioactivity in the esterified lipids was low, but radioactivity was found mainly in the diacylglycerols, phosphatidic acids and diacyl *sn*-glycero-3-phosphorylcholine (GPC). At 40 min after injection radioactivity of the esterified lipids attained a maximum. During this time interval radioactivity was distributed mainly in the diacyl GPC, diacyl *sn*-glycero-3-phosphorylethanolamine (GPE) and to a lesser extent in the inositol phosphoglycerides and triacylglycerols. At all times the radioactivities of the acyl groups of alkenyl acyl GPE and serine phosphoglycerides were quite low.

The specific radioactivity of individual glycerides is expressed as dpm/nmol of the oleoyl group in the respective glycerides in Table IV. The diacylglycerols and triacylglycerols had higher specific radioactivities than the phosphoglycerides, and maximum specific radioactivities for the diacylglycerols and triacylglycerols were achieved at 3 and 20 min, respectively. Although the specific radioactivity of the phosphatidic acids was highest at 1 min after injection, its level was much lower than the diacylglycerols. Specific radioactivities of the diacyl GPC and diacyl GPE increased with time, and the rate of increase for these two phosphoglycerides was nearly constant. Among the phosphoglycerides, inositol phosphoglycerides had the highest specific radioactivity, and radioactivity attained a plateau at 10 min after injection. Also the rate of increase in specific radioactivity for the inositol phosphoglycerides seemed to be more rapid than the diacyl GPE and diacyl GPC.

DISCUSSION

Results from this experiment confirm our earlier findings that long chain fatty acids are metabolized rapidly in the brain in vivo (1,2).

TABLE IV

Specific Radioactivities (dpm/nmol Oleoyl Group) of Neutral Glyceride and Phosphoglyceride Classes in Mouse Brain after Intracerebral Injection of [1-^{14}C]-Oleic Acid[a]

Min after injection	DG	TG	PA	IPG	SPG	Diacyl GPC	Diacyl GPE	Alkenyl acyl GPE[b]
1	905	34	13.4	5.0	1.5	1.0	1.4	0.3
3	2480	112	8.6	15.0	1.5	2.0	3.6	0.6
6	1550	326	5.5	21.9	0.7	4.7	7.0	0.9
10	1810	418	6.9	34.4	---	6.8	11.8	1.2
20	912	432	---	32.9	2.2	8.9	15.1	1.7
40	417	404	6.0	34.4	2.1	10.7	14.5	1.7
80	384	220	6.1	37.5	3.0	11.1	17.4	1.5

[a]Amounts of oleoyl groups (nmol/brain) in neutral glycerides and phosphoglycerides were: DG, 0.008; TG, 0.049; PA, 0.23; IPG, 0.45; SPG, 1.17; diacyl GPC, 5.14; diacyl GPE, 1.58; acyl GPE, 1.10. Radioactivity of neutral glycerides and phosphoglycerides is assumed to be in oleoyl group. For abbreviations see Table III.

[b]Specific radioactivity of 18:1 acyl group of alkenyl acyl GPE.

The radioactivity of the free oleic acid decreased rapidly in brain with a half-life of ca. 5 min. The specific radioactivities of the phosphatidic acids and the diacylglycerols were maximum at 1 and 3 min after injection, respectively. The biosynthesis in vivo of the triacylglycerols, diacyl GPE and diacyl GPC through the phosphatidic acids and diacylglycerols as intermediates has been discussed previously (1). In the present experiment, we have also observed material in the aqueous upper phase exhibiting a maximum radioactivity at 6 min after injection (Table I). Although the exact nature of this material has not been elucidated, some of its properties resemble those of the long chain acyl CoA (22). However the transient increase and decrease in radioactivity of this material during these time intervals suggests that it may also be a protein-linked compound associated with the diacylglycerols. The involvement of a heat-stable, water soluble protein-bound intermediate for the biosynthesis of cholesterol has been reported (23). This intermediate is also resistant to the organic extraction procedure, a property similar to the unidentified material in the aqueous phase.

After intracerebral injections of [^{14}C]-oleic acid, radioactivity was incorporated rapidly into the triacylglycerols with a maximum attained between 10 and 20 min after injection. Although more radioactivity was incorporated into the phosphoglycerides than the triacylglycerols, the specific radioactivity of the triacylglycerols was 50 to 100-fold higher than the phosphoglycerides. The high specific radioactivity of the triacylglycerols is surprising since only trace amount of this lipid is present in the brain (24). Due to the higher specific radioactivity, the diacylglycerols gave a better precursor-product relationship with the triacylglycerols than the phosphoglycerides. Between 20 and 80 min after injection, we further observed that specific radioactivity of the triacylglycerols decreased almost 50%, whereas specific radioactivities of the diacyl GPC and diacyl GPE were still increasing. This difference in metabolic pattern suggests that the triacylglycerols may be turning over in brain faster than the phosphoglycerides. The difference can be attributed to the presence of different diacylglycerol pools in brain. Also triacylglycerols with high metabolic activity may be localized in special brain regions or cell types such as the endothelial cells. This has been demonstrated in an in vitro system by incubating the choroid plexus with labeled fatty acid precursors (4).

Among the phosphoglycerides, specific radioactivity of the inositol phosphoglycerides was higher than the diacyl GPE and diacyl GPC. Also radioactivity incorporated into the inositol phosphoglycerides was most rapid within the first 10 min after injection and reached a plateau afterwards. Specific radioactivities of the diacyl GPE and diacyl GPC behaved differently and increased steadily with time throughout the experiment. Although biosynthesis of the inositol phosphoglycerides through CDP-diglycerides has been demonstrated in brain in vitro (25,26), we have not been able to detect this intermediate under the conditions of the present experiment. Possible metabolic relationships between the inositol phosphoglycerides and the diacylglycerols has been suggested due to their similar acyl group compositions (27). However any hydrolysis of inositol phosphoglycerides to diacylglycerols would not be detected in the present experiment.

In comparison with palmitic acid (1), a greater proportion of radioactivity is incorporated into the diacyl GPE using oleic acid as the

precursor. This is not surprising since there is a larger endogenous content of oleoyl groups than palmitoyl groups with the diacyl GPE (18). Baker and Thompson (5) have demonstrated that differences in brain fatty acid metabolism in vivo may also be due to a positional specificity within the glyceride molecule. Palmitate is preferentially incorporated into the 1 position, whereas oleate is incorporated equally well in both the 1 and 2 positions. This may not apply to the acyl groups of the plasmalogens and serine phosphoglycerides, since very little radioactivity is incorporated into either of these lipid molecules. Although the alkenyl acyl GPE contains an appreciable amount of oleoyl group in the 2 position, the oleoyl groups in this phosphoglyceride appear to be metabolically different from the diacyl GPE.

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REFERENCES

1. Sun, G.Y., and L.A. Horrocks, J. Neurochem. 16:181 (1969).
2. Sun, G.Y., and L.A. Horrocks, Ibid. 18:1963 (1971).
3. Bernsohn, J., L.M. Stephanides and H. Norgello, Brain Res. 28:327 (1971).
4. Marinetti, G.V., A. Weindl and J. Kelly, J. Neurochem. 18:2003 (1971).
5. Baker, R.R., and W. Thompson, Biochim. Biophys. Acta 270:489 (1972).
6. Dhopeshwarkar, G.A., and J.F. Mead, Ibid. 187:461 (1969).
7. Dhopeshwarkar, G.A., and J.F. Mead, Ibid. 210:250 (1970).
8. Dhopeshwarkar, G.A., C. Subramanian and J.F. Mead, Ibid. 210:250 (1970).
9. Dhopeshwarkar, G.A., C. Subramanian, D.H. McConnell and J.F. Mead, Ibid. 255:572 (1972).
10. Lapetina, E.G., G.G. Lunt and E. DeRobertis, J. Neurobiology 1:295 (1970).
11. Abdel-Latif, A.A., and J.P. Smith, Biochim. Biophys. Acta 218:134 (1970).
12. Ansell, G.B., and S. Spanner, J. Neurochem. 15:1471 (1968).
13. Horrocks, L.A., Ibid. 16:13 (1969).
14. Lapetina, E.G., G. Rodriquez Delores Arnaiz and E. DeRobertis, Biochim. Biophys. Acta 176:643 (1969).
15. Dhopeshwarkar, G.A., C. Subramanian and J.F. Mead, Ibid. 248:41 (1971).
16. Mandel, P., and J.L. Nussbaum, J. Neurochem. 13:629 (1966).
17. Sun, G.Y., and L.A. Horrocks, J. Lipid Res. 14:206 (1973).
18. Sun, G.Y., and L.A. Horrocks, Lipids 3:91 (1968).
19. Folch, J., M. Lees and G.H. Sloane-Stanley, J. Biol. Chem. 226:487 (1957).
20. Horrocks, L.A., and G.Y. Sun, in "Research Methods in Neurochemistry," Vol. I, Edited by Rodnight and Marks, Plenum Press, New York, 1972, p. 223.
21. Gottfried, E.L., J. Lipid Res. 8:321 (1967).
22. Ullman, M.D., and N.S. Radin, Ibid. 13:422 (1972).
23. Ritter, M.C., and M.E. Dempsey, Biochem. Biophys. Res. Comm. 38:921 (1970).
24. Sun, G.Y., J. Neurochem. 17:445 (1970).
25. Possmayer, F., and K.P. Strickland, Can. J. Biochem. 45:63 (1967).
26. Benjamin, J.A., and B.W. Agranoff, J. Neurochem. 16:513 (1969).
27. Keough, K.M.W., G. MacDonald and W. Thompson, Biochim. Biophys. Acta 270:337 (1972).

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Preparation and Characterization of Mouse Intestinal Phospholipase

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ABSTRACT

Phospholipase has been prepared in a stable, partially purified form from the small intestine of mice infected with the tapeworm *Hymenolepis nana*. The enzyme(s) attacks diacylphosphatides with liberation of free fatty acids and a corresponding decrease in phospholipid phosphorus, without accumulation of lysophosphatides. The reactivity with various substrates is strongly influenced by their physical state, the presence of other lipids, proteins or detergents. The phospholipids of some biomembranes (mitochondria, microsomes, red cell ghosts) are readily hydrolyzed under customary reaction conditions. The ensuing biochemical, morphological and functional alterations have been documented. In contrast, the diacylphospholipids of the cell membrane (intact erythrocytes, *Pseudomonas aerunginosa*) are not accessible to phospholipase action unless some alteration of the integrity of the cell is induced by physical or chemical means (hemolysis, polymyxin). The enzyme is proposed as a tool for the investigation of biomembranes and as a model for the study of phospholipase activity.

INTRODUCTION

In recent years a number of animal tissues have been shown to contain enzymes capable of splitting phospholipids by hydrolysis of the fatty acid ester bonds. Various criteria and methods (positional specificity, substrate affinity, Ca^{++} requirement, pH optimum) have been used to characterize enzymes from different sources and resolve mixtures of these activities encountered in some tissues (1-3). The presence of phospholipases A and B in rodent intestine was first reported by Epstein and Shapiro (4) and has since been confirmed in many laboratories (5-9). Subbaiah and Ganguly have recently described the subcellular distribution of these enzymes in rat intestine (10).

This report deals with the preparation and biochemical characterization of a stable, partially purified form of the phospholipase found in particularly high concentrations in the small intestine of albino mice infected with the tapeworm *Hymenolepis nana* (11).

MATERIALS AND METHODS

Animals

Adult male mice (Charles River, CD-1), average body weight 20 g, were housed in groups of 20 per cage in quarters provided with temperature, humidity and light controls (7 A.M.-9 P.M.-7 A.M., light-darkness cycle). These animals were used for both induction of high intestinal phospholipase concentrations by worm infection and as sources of worm eggs to maintain a small colony of infected mice over the past 3 years.

Routinely the mice were infected with *Hymenolepis nana* eggs 1 week after arrival, each animal receiving 3000-5000 eggs in 0.5 ml water by gavage using a N1611 curved needle 18 GA x 1.5 (Biomed, Instrument Div., Popper & Sons, Inc., N.Y.). A high phospholipase content of the intestine, suitable for enzyme extraction and purification, is already present after 10-12 days and persists for at least 6 weeks (11). We have used most often 4-5 weeks infected mice for enzyme preparation as described in the results section. A slightly shorter time is optimal for the harvesting of eggs. For this purpose the lower third of the small intestine of six to eight mice infected 25-30 days earlier was removed, immersed in water in a Petri dish and cut longitudinally to disperse the contents by gentle shaking. The easily identifiable, white, thread-like worms were transferred with tweezers through three to four washes in water to remove gross contaminants, and finally collected in ca. 10 ml water. After transfer of the parasites and fluid to a small porcelain mortar, the eggs were freed by gentle grinding with a pestle followed by stirring for 10-15 min with the aid of a magnetic bar. Five microliters of the suspension were used for counting at 35x magnification and the concentration of the eggs was adjusted to 6000-10,000 eggs per milliliter. The yield from eight mice was sufficient for infecting 30 new animals by administering to each 0.5 ml of the continuously stirred suspension as described above. It is worth adding that harvesting of the parasites should not be postponed much later than the 30th day since the number of worms to be

found declines considerably by the 5th week, probably as a consequence of self-cure. The latter process is accompanied by the development of immunity to reinfection, and it is therefore important to avoid accidental transmission of the infection via bedding or non-sterilized drinking water bottles and cages, so that animals kept for weeks before infection do not become immune and give negative results. For the same reason, it is important to obtain mice from breeding colonies where *Hymenolepis nana* infection is totally absent. In our experience the source mentioned above has met this requirement over the past 3 years.

Substrates

The substrates of phospholipase, their sources and preparation methods are listed below.

Phosphatidylcholine (PC): PC was prepared from egg yolk according to Ansell and Hawthorne (12). The silicic acid column eluate in chloroform-methanol 7:3 was added with 0.005% of the antioxidant 4-methyl-2-6-di-tert-butyl phenol and brought to dryness in a rotary evaporator. After flushing with nitrogen the PC was resuspended in water to a final concentration of 20 μmol/ml and stored frozen in 5-10 ml aliquots at -15 C. For use, the frozen samples were thawed in lukewarm water. According to the particular experimental design, the substrate was mixed directly with buffer or dispersed by addition of Triton X-100 or sonicated in a Raytheon Model DF101 magnetostrictive oscillator, 10 KC, for two successive 3 min intervals.

Phosphatidylethanolamine (PE): PE was obtained from egg yolk according to Ansell and Hawthorne (13) and processed for storage and use as indicated above for PC.

Lysophosphatidylcholine (LPC): LPC was prepared from PC using snake venom according to Long and Penny (14). After extraction with hot alcohol, the phospholipid was dried at reduced pressure and redissolved in a small volume of water (60-80 μmol/ml). The solution was dialyzed overnight against distilled water and the concentration adjusted to 20 μmol/ml, for storage at -15 C. The clear solution resulting upon melting in lukewarm water was used routinely without further treatment.

Soybean phosphatides (Asolectin): The mixture of phosphatides was obtained from a commercial source (Associated Concentrates, Inc., Long Island, N.Y.). When used as a substrate the material was dispersed in water (5 mg/ml) by homogenization in a motor driven Teflon-glass homogenizer. If needed, sonication was carried out as indicated for PC. Microdispersions of Asolectin for use in the reactivation of beef heart mitochondrial enzymes were prepared and stored according to Fleischer and Fleischer (15).

Rat liver mitochondria and microsomes: These were obtained by the standard preparation methods in isotonic sucrose and were stored at -15 C in a sucrose volume corresponding to 50% of the liver weight. Lipid and protein fractions of the two preparations were obtained by extraction with aqueous acetone ammonia according to Fleischer and Fleischer (16). One volume of mitochondrial or microsomal suspension was mixed with 24 volumes of acetone-water-ammonia 22.5:1.5:0.004 v/v/v and the mixture separated at 800 x g for 10 min. The pellets were then washed three times with isotonic sucrose, sedimented at 20,000 x g for 10 min and resuspended in the same medium in one-half the original volume. These preparations, containing 5.1 and 15.3 mg of protein per milliliter, respectively, are here referred to as mitochondrial and microsomal protein. The corresponding acetone supernatants were evaporated under reduced pressure, resuspended in twice the original (mitochondrial or microsomal) volume of water and extracted according to Bligh and Dyer (17). Each chloroform extract was added with 0.005% of the antioxidant 4-methyl-2,6-di-tert-butyl phenol, brought to dryness under reduced pressure, and the lipid residue was dispersed in one half the original volume of sucrose giving a suspension containing 15.3 and 6.3 μmol phospholipid per milliliter mitochondrial and microsomal extract, respectively. All preparations were stored at -15 C and simply thawed before use unless otherwise indicated.

Red cell ghosts: Washed human erythrocytes were lysed in 0.01 M Tris buffer pH 7.4 and washed free of hemoglobin by repeated centrifugation at 14,000 x g for 10 min in the same medium. The cells were resuspended in eight-tenths of the original volume and incubated with phospholipase as specified in the results section.

Pseudomonas aeruginosa: The bacteria were grown for 24 hr on Difco Nutrient Broth, harvested by centrifugation, washed with water three times and finally resuspended in water. Cell concentration was adjusted so that, upon undergoing standard operations of incubation and further dilution, the control sample in water would give an optical density reading of 0.13-0.16 at 500 λ in a Coleman junior photometer. In the experiments reported here, the cells were first incubated at room temperature with phospholipase and polymyxin; a 2 ml aliquot was withdrawn at a chosen time and the

initial OD reading was taken. Then 0.2 ml of 2 M NaCl was added, rapidly mixed, and the cell's response was followed by short interval readings of the OD changes. Cells incubated without phospholipase served as controls.

Enzyme Assays

Phospholipase activity: Reaction conditions varied to fit specific experimental goals. The following buffers were used at 0.1 M strength for the pH studies: acetate, MES, Tricine. Routinely 1 volume buffer was mixed with 0.5 volumes substrate in aqueous medium at 37 C, and the enzyme preparation was added in microliter amounts to start the reaction. The standard assay for lysophospholipase activity used 0.1 M phosphate buffer pH 6.6 added with 0.5 volumes of 2 x 10^{-2} M lysolecithin. Hydrolysis of the various substrates was determined by one of two basic procedures: (a) extraction of the unreacted phospholipid according to Bligh and Dyer (17) and estimation of the P content of the chloroform phase (Fiske and Subbarow [18] and Barlett [19]); (b) extraction and titration of the liberated fatty acids in heptane according to Dole (20). The first procedure was most useful when diacylphospholipids were studied, while the second was preferred when LPC was the substrate.

Quantitative estimates involving both fatty acids and phospholipids were usually performed on Bligh and Dyer extracts. To obviate the interference with fatty acid titration normally encountered when diacylphospholipid are present, an aliquot of the chloroform phase was first dried, the lipid redissolved in 3-4 ml dry chloroform and loaded onto a small silicic acid column (at least 1 g/2.5 μmol extracted phospholipid). The column was then washed with 3 volumes of dry chloroform, and the combined eluates dried under reduced pressure. The residue was dissolved in heptane and the fatty acids titrated with 0.01 N NaOH. This procedure, which works well with pure substrates, was controlled using known amounts of pure fatty acids. When very small quantities were involved, the fatty acid estimation method of Mahadevan et al. (21) was followed.

Aliquots of the Bligh and Dyer extracts were also used for thin layer chromatography on silica plates (Eastman, Chromagram) following the two step procedure of Skipski and Barclay (22). Visualization of the phospholipid spots was achieved by spraying with dichlorofluorescein and viewing in UV light.

Glycerylphospholycholine (GPC) was estimated by the ennaiodide method of Appleton et al. (23) adapted for PC and lysoPC preparations (24). The formation of glycerylphosphorylethanolamine and GPC in red cell ghosts suspensions was detected by paper chromatography according to Dawson et al. (25).

Oxidative enzymes of beef heart mitochondria: Standard techniques were followed for the estimation of succinate-cytochrome reductase (26) of heavy beef heart mitochondria incubated at 30 C with mouse phospholipase.

RESULTS

Extraction and Purification of Mouse Phospholipase

The procedure developed and finally adopted in our laboratory for preparation of mouse intestinal phospholipase consists of the steps described below. The volumes and amounts given refer to a preparation run using eight mice; a larger number of animals can be processed with a proportional increase in fluid and reagents. Estimation of phospholipase B activity was used to follow the various stages of purification.

Intestinal homogenate: Mice infected with *Hymenolepis nana* 4-5 weeks earlier are anesthetized with ether and killed by decapitation. The entire small intestine is dissected, cut in 4-5 segments and the content extruded by gentle pressure along the peritoneal surface. After weighing, the tissue is minced with scissors and homogenized in 19 volumes ice cold medium (8% sucrose, 1 x 10^{-2}M Tris pH 7.4, 1 x 10^{-3}M Mg, 1 x 10^{-3}M dithiothreitol) with the aid of a motor driven Teflon-glass homogenizer. The homogenates are pooled (280 ml) and centrifuged at 8000 x g for 10 min. (For this and all the following centrifugation steps except dialysis and storage, we have found it advantageous to use a #30 Spinco rotor and 38.5 ml tubes.) The supernatant (230 ml) contains 80-90% of the total homogenate activity.

Precipitation with Protamine: The 8000 x g supernatant is added slowly, with stirring, with 0.25 volume of protamine sulfate (Calbiochem., 1% in water at room temperature) and the mixture is allowed to stand in the cold for 20 min prior to centrifugation (25,000 x g x 15 min). The whitish pellets contain the bulk of the phospholipase activity.

Extraction with Tris-Triton X-100: To each centrifuge tube are added in succession 5 ml 0.3 M Tris pH 7.4 (containing 1 x 10^{-3}M dithiotreitol) and 0.5 ml 1% Triton X-100. The pellet is first dispersed with the aid of a glass rod, and the suspension is diluted with 15 ml water containing 1 x 10^{-3}M dithiothreitol and transferred to an ice cold beaker. Washing of the centrifuge tubes with an additional 20 ml of Tris-water medium aids to recover all the particulate material, and extraction is com-

TABLE I

Purification of Mouse Intestinal Phospholipase[a]

Preparation	Total activity	%	Activity/mg protein
8000 x g supernatant	402,000	100	342
Protamine-Triton X extract	220,000	54	1123
40% Ammonium sulfate, ppt	213,000	53	1627
60% Ammonium sulfate, ppt	163,000	40	2634
Calcium phosphate gel eluate–I	60,000	15	6024
Calcium phosphate gel eluates I + II	140,000	34	4740

[a]Activity values represent micromoles lysolecithin hydrolyzed per hour under standard assay conditions.

pleted by homogenization with a few hand strokes in a Teflon-glass homogenizer. Centrifugation at 25,000 x g for 15 min gives a lightly colored supernatant containing ca. 50% of the intestinal homogenate activity. The partial recovery at this step is due to difficulties in solubilizing the enzyme from the protamine precipitate rather than to inhibition by the detergent, which, at the above concentrations, does not affect the total phospholipase B activity.

Fractionation with ammonium sulfate I and II: The supernatant of the preceding step (140 ml) is mixed slowly, stirred with 1 volume cold 80% saturated ammonium sulfate and allowed to stand in the cold for 15-20 min. The light precipitate thus formed is separated at 25,000 x g for 15 min, and the slightly cloudy supernatant is slowly added with 0.5 volume cold saturated ammonium sulfate (final saturation, 60%). After 30 min in the cold, a well defined precipitate is present and is easily separated by centrifugation for 15 min at 25,000 x g.

Fractionation on calcium phosphate gel: The pellets of the preceding step are dissolved in 40 ml cold 0.1M Tris pH 7.4, 10^{-3}M dithiothreitol, and 12 ml calcium phosphate gel suspension (27) is added. After mixing, centrifugation at 2000 x g for 10 min separates the gel, which is washed twice with 60 ml 1% sodium chloride, 1 x 10^{-3}M dithiothreitol, and is finally extracted with 20 ml (alternatively twice with 10 ml aliquots) 0.4M potassium phosphate buffer pH 7.4.

Dialysis and storage: The gel eluate is dialyzed overnight against two changes of distilled water (8 liters), and the clear solution is stored frozen at -15 C. The results of one experimental run are given in Table I.

The procedure detailed above gives preparations averaging 1 mg protein and 3500-6000 units lysophospholipase activity per milliliter; no loss of activity in the frozen state is detectable over an eight month period. Alternatively, the dialyzed preparation can be freeze-dried and kept at -15 C as a powder. Lyophylization is accompanied by a 50% loss of activity, but the preparation is stable afterwards over a

TABLE II

Effect of Reaction Conditions on Phospholipase Activity

Substrate[a]		Treatment or addition			
		None	Sonication[b]	Protein[c]	Triton X-100[d]
Phosphatidylethanolamine	pH	5.2-5.9	5.2	5.2-6.6	
	V	95	110	155	0.0
Phosphatidylcholine	pH		5.2		6.99
	V	0.0	105	0.0	117
Asolectine	pH	6.2	6.2	6.2	6.6
	V	45	12	87	109
Lysolecithin	pH	5.2-7.3		5.2-7.3	
	V	418		432	

[a]Substrate concentration: phosphatidylethanolamine 1.2 x 10^{-3}M; phosphatidylcholine = 1.3 x 10^{-3}M; asolectine = 1.1 x 10^{-3}; lysolecithin = 1.4 x 10^{-3}M. For each substrate the pH value or range indicated is that at which reaction velocity (V) is highest. V is given as millimicromoles of substrate hydrolyzed per hour by 1 μg lyophilized enzyme.

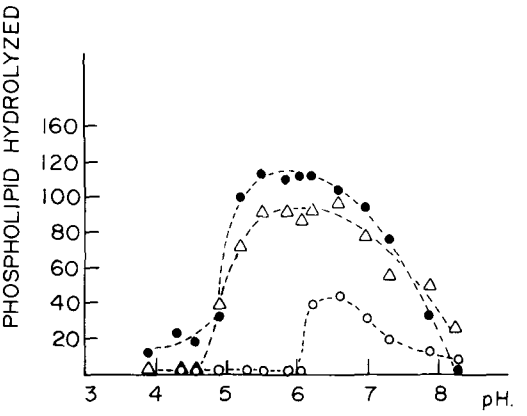

FIG. 1. Hydrolysis of mitochondrial phospholipids of rat liver by mouse phospholipase. Hydrolysis rate expressed on abscissa as millimicromoles phospholipids hydrolyzed per hour by 1 μg lyophilized enzyme reacted with intact rat liver mitochondria (●—●), their lipid extract (○—○), and lipid extract added with mitochondrial protein (△—△). Phospholipid concentration in three series = 1.4; 1.6; and 1.6 μmol/ml, respectively; added protein = 1.01 mg/ml.

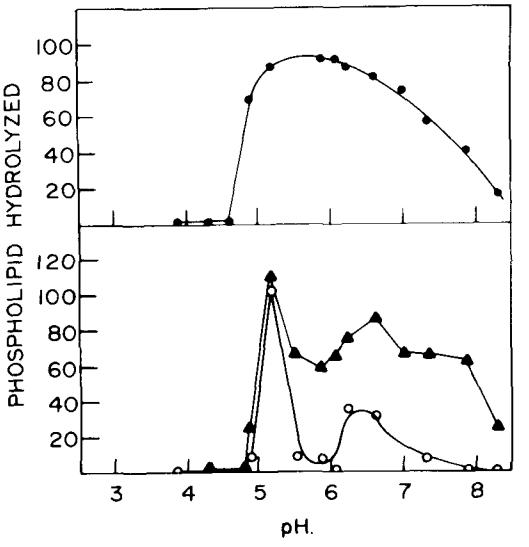

FIG. 2. Hydrolysis of microsomal phospholipids of rat liver by mouse phospholipase. On abscissa hydrolysis rate expressed as millimicromoles phospholipid hydrolyzed per hour by 1 μg lyophilized enzyme reacted with: intact rat liver microsomes (●—●); their lipid extract (○—○); lipid extract added with microsomal protein (▲—▲). Phospholipid concentration in three series = 1.96; 2.39; and 2.39 μmol/ml, respectively. Added microsomal protein = 600 μg/ml.

prolonged period of time (more than 1 year in our experience).

For testing with the various substrates the enzyme is best prepared in relatively concentrated solutions (0.5-1 mg protein/ml), since rapid loss of activity follows dilution in water at concentrations below 200 μg protein per milliliter. Addition of electrolytes or serum albumin is without effect, and only partial stabilization is achieved by the presence of SH groups (2 x 10^{-3}M dithiothreitol). We have routinely used microliter volumes of freshly thawed material added to pre-equilibrated mixtures of buffers and substrates with good reproducibility of results.

Further purification of the enzyme by gel filtration or ion exchange chromatography (Deae-Sephadex) is impractical, since little or no gain in specific activity is obtained by either procedure. Gel electrophoresis shows the presence of seven distinct bands (four major, three minor), two of which are associated with phospholipase activity recoverable from the sliced gel by extraction with glycerol buffer. Peak activity coincides with a band corresponding to 25% of the stained protein in the gel.

Substrates

Mouse intestinal preparations readily hydrolyze the diacylphospholipids of mitochondria, microsomes, red cell ghosts and bacterial membranes, and attack with varying affinity the individual phospholipids reacted in the form of aqueous dispersions. The hydrolytic reaction and the attendant biochemical characteristics (pH optima, apparent Vmax) are strongly influenced by the physical state of the substrates and the presence of nonlipid material (Figs. 1-5, Table II).

Figure 1 shows the rather broad range of pH values at which the phospholipids of intact rat liver mitochondria are hydrolyzed. Separation of the lipid moiety by extraction with aqueous acetone-ammonia results in a considerable reduction of reaction rate and pH range with a shift of the optimum toward the neutral region. Addition of the protein residue to the lipid extract restores the pH curve to a range and height comparable to those seen with intact mitochondria (Fig. 1). Similar results are obtained with liver microsomes (Fig. 2).

Recombination experiments of the type described above indicate that restoration of the protein to phospholipid ratio present in the intact organelles is not essential, since one-third of the equivalent protein is sufficient to produce a maximal effect on both hydrolysis rate and pH range. In this respect the mitochondrial protein is more effective than the corresponding microsomal preparation on a protein weight basis, even when microsomal phospholipids are the substrate.

Partially purified phospholipid mixtures (Asolectin) and individual phospholipids behave

TABLE III

Stoichiometry of Reaction Products during Hydrolysis of Phospholipids by Mouse Phospholipase[a]

Substrate	-ΔPLP-P	+FA	FA/P	Reaction conditions or additions
Egg lecithin	3.03	5.91	1.95	Sonicated, pH 5.3
	3.34	6.82	2.04	Sonicated, pH 5.3
	0.84	1.72	2.05	pH 6.99, Triton-X
Egg-phosphatidylethanolamine	2.53	4.75	1.88	pH 6.23 Mitochondrial protein
Rat liver mitochondria	3.88	7.38	1.90	pH 6.23
	4.68	9.02	1.93	pH 6.23

[a]Values in columns headed -ΔPLP-P and +FA indicate, respectively, micromoles of phospholipid hydrolyzed and of fatty acid liberated under reaction conditions specified in last column. Table was compiled from experimental data obtained in a number of experiments using various enzymatic preparations of different specific activity.

similarly. Addition of protein or detergent or sonication of the substrate can greatly influence the rate of the hydrolytic reaction, although no single experimental variable induces similar effects on all substrates tested, thus underlining the complexity of the interactions involved in initiation and enhancement of the enzymatic attack. For example, addition of mitochondrial protein augments both reaction rate and pH range for PE and Asolectin suspensions (Fig. 3, Table II) but has no effect on PC, which becomes susceptible to hydrolysis only after dispersion by sonication or by addition of Triton-X (Fig. 4). These procedures, by contrast, reduce the reactivity of the other two substrates as shown in Table II. The presence of two pH optima for PC after addition of Triton X-100 has been confirmed in a number of experiments similar to those shown in Figure 4.

Among the substrates tested, lysophosphatidylcholine shows the highest reactivity over a broad pH range unaffected by addition of protein (Fig. 5), detergent or sonication; hence it was selected as the substrate of reference during the development of the enzyme purification procedure. Other substrates found susceptible to mouse phospholipase include purified phosphatidic acid and the phospholipids of bacterial membrane vesicles (28-31).

Reaction Products

Phospholipid hydrolysis by the mouse phospholipase results in liberation of fatty acids and a concomitant decrease in phospholipid P. As shown in Table III the molar ratios of fatty acid to P are quite uniform under widely different experimental conditions and indicate that the

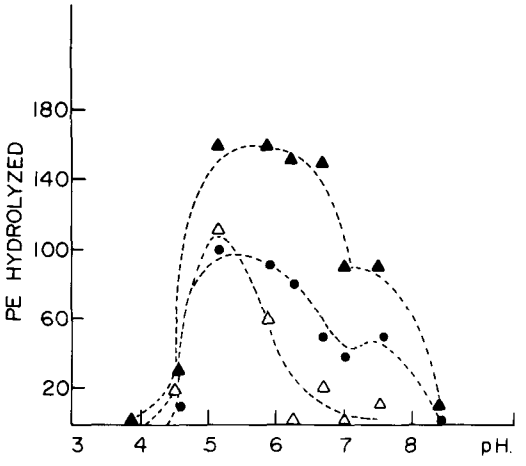

FIG. 3. Hydrolysis of phosphatidyl ethanolamine by mouse phospholipase. On abscissa reaction rate expressed as millimicromoles phosphatidylethanolamine hydrolyzed per hour by 1 μg lyophilized enzyme reacted with 1.18 μmol/ml substrate in aqueous dispersion (●—●); after sonication (3 x 3' at 10 KC) (△—△); after addition of mitochondrial protein (335 μg/ml) (▲—▲).

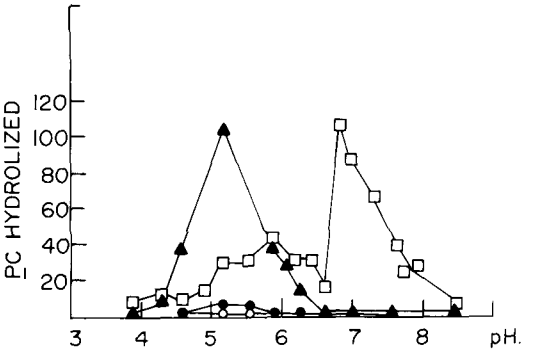

FIG. 4. Hydrolysis of phosphatidylcholine by mouse phospholipase. Hydrolysis rate expressed on abscissa as millimicromoles phosphatidylcholine hydrolyzed per hour by 1 μg lyophilized enzyme reacted with phosphatidylcholine (1.33 μmol/ml) in aqueous dispersion (○—○); after sonication (3 x 3' at 10 KC) (▲—▲); after addition of mitochondrial protein (335 μg/ml) (●—●); after addition of triton X (8.3 μl/ml) (□—□).

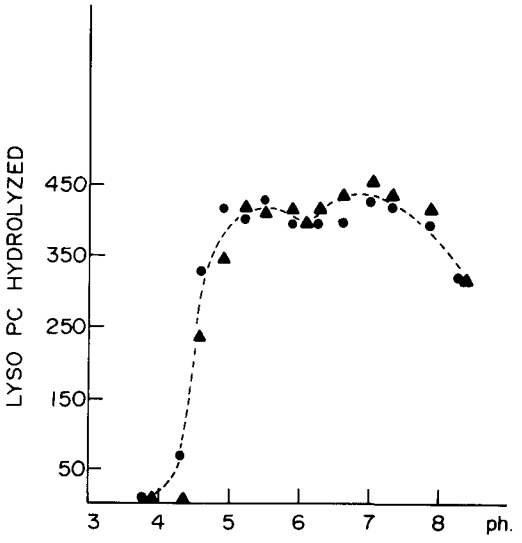

FIG. 5. Hydrolysis of lysophosphatidylcholine by mouse phospholipase. Hydrolytic rates are given as millimicromoles of lysophosphatidylcholine hydrolyzed per hour by 1 μg lyophilized enzyme reacted with lysophosphatidylcholine (1.44 μmol/ml) in aqueous dispersion (●—●); or after addition of mitochondrial protein (500 μg/ml) (▲—▲).

diacylphospholipids are hydrolyzed without accumulation of detectable amounts of lysophospholipids. The absence of a lysolecithin spot in thin layer chromatograms of reaction samples withdrawn at different stages of the hydrolytic reaction with PC supports this conclusion.

Hydrolysis of PC and lysoPC is accompanied by the accumulation of acid soluble P and glycerylphosphorylcholine (GPC). Table IV shows the quantitative relationship between these products.

Qualitative analysis by paper chromatography confirmed the presence of both GPC and its ethanolamine-containing analog, GPE in red cell ghosts incubated with mouse phospholipase.

Attempts at separation of phospholipase A and B activities by the addition to PC of deoxycholate over a wide range of concentrations were unsuccessful due to a concurrent inhibition of the hydrolytic reaction as a whole. At present no indication that the two activities can be separated has been obtained, despite the variety of experimental conditions employed, including the multiple steps of the purification procedure as shown in Table V.

Action on Biological Membranes

The mouse intestinal phospholipase was tested for its action on a number of biological membranes to ascertain its possible use as a tool in special experimental conditions, as is current

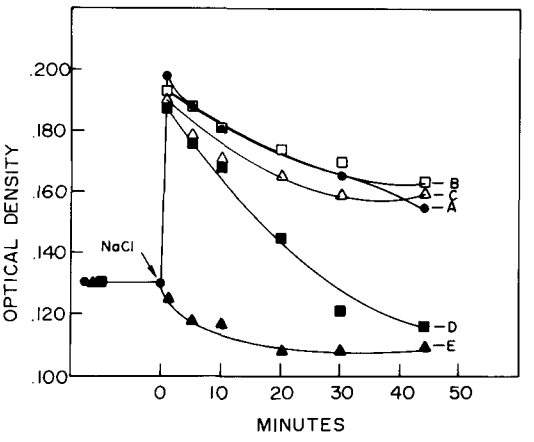

FIG. 6. Response to osmotic challenge of *Pseudomonas aeruginosa* cells after treatment with phospholipase. ○—○ Control cells; □—□ cells + polymyxin, 5 μg/ml; △—△ cells + polymyxin, 10 μg/ml; ■—■ cells + polymyxin, 5 μg/ml, + phospholipase, 11 μg/ml; ▲—▲ cells + polymyxin, 10 μg/ml, + phospholipase, 11 μg/ml.

practice with other phospholipases (A_1, A_2, C, D). Intact bacterial cells, red blood cells and their ghosts and beef heart mitochondria were selected for this purpose, and the results are summarized below.

Bacterial cells: Incubation of intact bacterial cells (*Pseudomonas aeruginosa*) with mouse phospholipase is without effect on bacterial phospholipids, although the latter are rapidly hydrolyzed when suspensions of cells disrupted by sonication are exposed to the enzyme. The apparent protection of the phospholipids from enzymatic attack in the intact cell can be removed by treatment with relatively low concentrations of Polymyxin, known to induce definite changes in bacterial membrane morphology (33). The two agents appear to exert cumulative effects with regard to alterations in cell function, since their combination causes a considerable impairment in the ability of the cells to react to osmotic challenge. Figure 6 (curve A), shows the characteristic response of normal cells to an increase in tonicity consisting of (a) a rapid decrease in cell size which is reflected in the sharp increase in optical density readings, followed by (b) a slow swelling process extending over 40 min and measured by the slow decline of the OD curve. While addition of polymyxin alone is without effect (curves B and C), combination with phospholipase causes lowering of the maximum OD value and acceleration of the swelling process in proportion to the amount of polymyxin (curves D and E). According to the evidence presented by Bernheim (34-36), these changes represent alterations in the permeability of bacterial

TABLE IV

Glycerylphosphorylcholine Formation from Phosphatidylcholine and Lysophosphatidylcholine Hydrolyzed by Mouse Phospholipase

Substrate[a]	Reaction products[b] +FA or -ΔPLP-P	ASP	GPC
Phosphatidylcholine	0.76	0.78	0.70
	1.14	1.13	1.16
Lysophosphatidylcholine	—	1.48	1.55
	—	2.55	2.92
	4.11	4.22	4.05

[a]Reaction conditions: (a) phosphatidylcholine (sonicated) 1.6 x 10^{-3}M, acetate buffer pH 5.2 0.066M, enzyme 27 μg/ml; (b) lysophosphatidylcholine 6.6 x 10^{-3}M, tricine buffer pH 6.6 0.066M, enzyme 8 μg/ml.

[b]Reaction products expressed as micromoles of hydrolyzed phospholipid (-ΔPLP, for phosphatidylcholine; +FA for lysophosphatidylcholine and of released acid soluble P (ASP) and glycerylphospholycholine (GPC).

TABLE V

Hydrolytic Activity of Mouse Intestinal Preparations on Diacyl- and Monacylphospholipids[a]

Preparation	Substrate PC	LysoPC	PC/lysoPC
Homogenate	61	690	.088
8000 x g x 10' supernatant	81	841	.096
Tris-Triton extract from protamine, ppt	75	625	.120
60% Ammonium sulfate, ppt	97	1370	.071
Calcium phosphate gel eluate	267	3624	.078

[a]Hydrolytic activity of various preparations of mouse intestinal mucosa reacted with phosphatidylcholine (PC) and lysophosphatidylcholine (lysoPC) is given as micromoles hydrolyzed per hour per milligram protein. Values of last column are ratios of activities for two substrates.

membranes due to loss of selectivity to permeants.

Work done in other laboratories (28-31) using mouse phospholipase preparations supplied by us has confirmed the susceptability of bacterial membrane phospholipids to attack by this enzyme.

Red blood cells: Purified mouse phospholipase is without effect on the phospholipids of red cells from various species (man, rat, sheep, pig) incubated in conditions preventing hemolysis. It should be emphasized that crude intestinal homogenates contain a lytic factor, which is effectively separated from the phospholipase during the calcium phosphate gel absorption step. Enzyme preparations preceding this treatment cause hemolysis and hydrolysis of RBC phospholipids, developing with apparently identical time courses in the initial stages of the reaction. Later preparations are inactive.

In contrast with the lack of action on intact red cells, the mouse phospholipase readily hydrolyzes the phospholipids of red cell ghosts with liberation of free fatty acids and a corresponding decrease in phospholipid P.

The hydrolytic process is accompanied by a profound alteration of the ghost morphology as revealed by freeze fracture electron micrographs. The normal pattern of distribution of particles on the internal fracture face of the ghosts consisting of small chains or clusters is shown in Figure 7A. After treatment with mouse phospholipase at different concentrations, areas of denudation and formation of large clusters are seen (B,C) indicating a rearrangement of the constitutive elements of the ghost membrane. The morphological changes show some similarity to those observed by Weinstein and Wallach (37) after treatment of RBC ghosts with phospholipase C.

Beef heart mitochondria: Rapid hydrolysis of the diacylphospholipids of these preparations occurs upon incubation with small concentrations of mouse phospholipase. Respiratory activity (succinate-cytochrome C reductase) is inhibited and can be restored by addition of microdispersed phospholipids. No accumulation of lysophospholipids can be detected and the necessity of multiple washes with albumin, as is the case with snake venom phospholipase, is obviated.

DISCUSSION

The results reported here show the presence in the intestine of *Hymenolepis nana*-infected mice of an active phospholipase, capable of attacking all the major diacylphospholipids with liberation of free fatty acids and the corresponding glycerylphosphoryl fragments. In the following discussion the question of classification and other special aspects of the activity of the enzyme will be considered.

Current terminology identifies the acylhydrolases acting on diacylphosphatides on the basis of their positional specificity as phospholipases A_1 and A_2, and designates the enzymes acting on monoacylphosphatides (lysophospholipids) as lysophospholipases (E.C. 3.1.1.5.). This classification does not include all possible modes of enzymatic attack and McMurray and Magee (1) have recently proposed the term "phospholipase B" (hitherto used for the E.C. 3.1.1.5.) for "the enzyme which presumably hydrolyzes both of the acyl ester linkages in diacylphosphatides simultaneously." The same

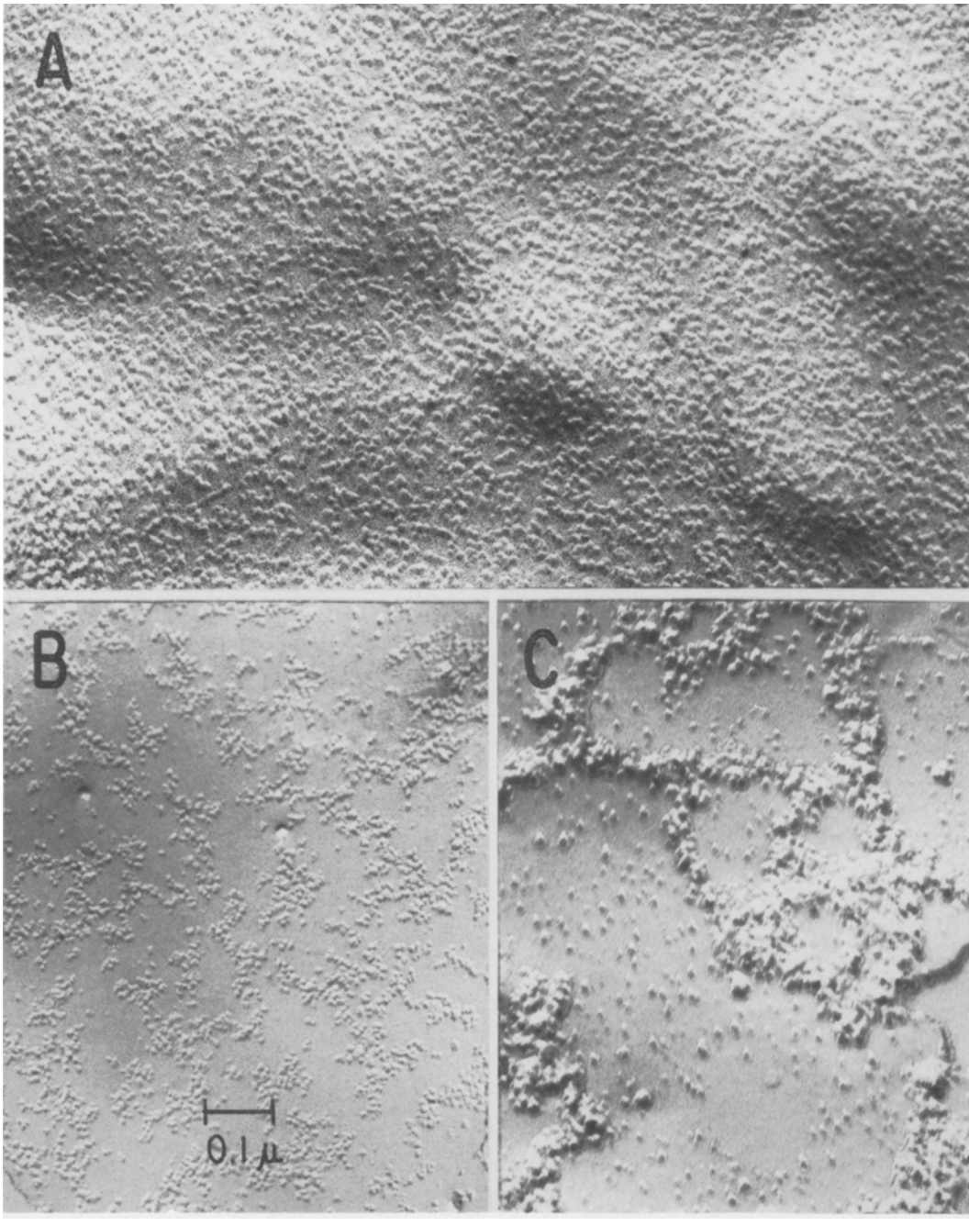

FIG. 7. Action of mouse phospholipase on red cell ghosts. A = Control RBC ghosts; B,C = RBC ghosts incubated for 45 min with 120 and 360 μg phospholipase per milliliter, respectively.

authors point out that the presence of several acylhydrolases in the same tissue often complicates the task of identifying single enzymatic activities. Such might be the case in the experiments reported here, in which a separation of activities (diacyl- and monoacylhydrolases) did not occur throughout the sequence of purification or after addition of deoxycholate. The negative results with the latter procedure are particularly significant, since this is the usual method for demonstrating in vitro the presence of the A_2 type of enzyme. Differentiation on the basis of pH optima is infirmed by the dependence of this parameter on such

factors as substrate dispersion and the presence of other materials. The failure to identify two enzymatic activities can be interpreted for the time being as an indirect indication of the presence in mouse intestine of a hydrolase acting on both acyl ester bonds (type B of McMurray and Magee). This is, admittedly, a working hypothesis to be tested directly by further studies.

While the presence of yet another phospholipase in the intestine is not surprising, in view of the multiplicity of enzymes of this type found in many tissues, it is of considerable interest that such high levels should develop following worm infection. An accompanying short communication (11) details the actual quantities, the time course and the distribution of the enzyme in the intestinal tissue during the various phases of the infection. It suffices to point out here that in the areas of highest concentration, phospholipase activity can be readily shown using as little as 20-50 μg tissue, ca. 100 times less than the amount needed when other organs or normal intestines are used. It seems reasonable to infer that the pronounced increase in phospholipase activity has some yet unknown relationship to either the local injury or the development of a protective barrier against the parasite. In either case, a novel and unsuspected function for the enzyme is suggested.

The mouse intestinal phospholipase exhibits the wide variation in pH optima and the dependence on the physical state of the substrate that has been reported for almost all enzymes of this class, irrespective of their sources (36-39). As already pointed out, the conditions that maximize the hydrolytic activity differ considerably for the various substrates and no single experimental variable applies equally to all. Often inversion of effect is seen in passing from one substrate to another; this is true with regard to both the physical state of the substrate (sonication) or the addition of dispersing material (detergent, protein). The effect of protein normally associated with phospholipids in cell organelles is clearly shown in the experiments using lipid extracts from mitochondria and microsomes and some of the purified substrates. The fact that lipid mixtures are more readily attacked than their purified components indicates the importance of multiple lipid-lipid and lipid-protein interactions.

Another aspect of the relationship between enzymatic attack and the special state of the phospholipids is revealed in the experiments using erythrocytes and *Pseudomonas aeruginosa*. Our results are in agreement with reports, published during the course of our work (41-44), showing the virtual inaccessibility of the membrane phospholipids to acylhydrolases as long as the membrane structure is intact. Hemolysis (or pretreatment with polymixin in the case of the Pseudomonas cells) is sufficient to expose the phospholipids to the action of the enzyme. The morphological alterations of the RBC membrane suggest a mobilization of some of its components after hydrolysis of the phospholipids, as already shown to be the case for the phospholipase C-treated ghosts (45).

It is of interest that in the bacterial cells the biochemical lesion is accompanied by a measurable alteration in function involving loss of permeant selectivity. Other workers (28-31) have used our enzyme to digest phospholipids of membrane vesicles of *E. coli* and have shown a differential effect on catalytic activities associated with transport and the ability of the cells to retain transported solute.

The general susceptibility of phospholipids of cellular organelles and of exposed membranes to the action of the enzyme is also confirmed by the effect on beef heart mitochondria. We have already reported elsewhere the alterations in nerve function associated with treatment of the squid giant axon with phospholipase (46).

In conclusion, a broad spectrum of activity has been shown for this enzyme, which is now available in a stable, partially purified form, technically useful for further study as a model of phospholipase action and as a tool for the investigation of biological membranes.

ACKNOWLEDGMENTS

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REFERENCES

1. McMurray, W.C., and W.L. Magee, Ann. Rev. Biochem. 41:129 (1972).
2. Hanahan, D.J., "The Enzymes," Vol. V, Edited by P.D. Boyer, Academic Press, New York, 1971, p. 71.
3. Van Golde, L.M.G., R.N. McElhaney and L.L.M. Van Deenen, Biochim. Biophys. Acta 231:245 (1971).
4. Epstein, B., and B. Shapiro, Biochem. J. 71:615 (1959).
5. Marples, E.A., and R.H.S. Thompson, Ibid. 74:123 (1960).
6. Robertson, A.F., and W.E.M. Lands, Biochemistry 1:804 (1962).
7. Ottolenghi, A., J. Lipid Res. 5:532 (1964).

8. Gallai-Hatchard, J.J., and R.H.S. Thompson, Biochim. Biophys. Acta 98:128 (1965).
9. *Ottolenghi*, A., *Lipids 5*:531 (1970).
10. Subbaiah, P.V., and J. Ganguly, Biochem. J. 118:233 (1970).
11. Ottolenghi, A., Lipids 8:426 (1973).
12. Ansell, G.B., and S.N. Hawthorne, "Phospholipids," Elsevier Publishing Co., Amsterdam, 1964, p. 87.
13. Ansell, G.B., and S.N. Hawthorne, Ibid., p. 99.
14. Long, G., and I.F. Penney, Biochem. J. 74:123 (1960).
15. Fleischer, S., and B. Fleischer, "Methods in Enzymology," Vol. X, Academic Press, New York, 1967, p. 423.
16. Fleischer, S., and B. Fleischer, Ibid., p. 412.
17. Bligh, E.G., and W.J. Dyer, Can. J. Biochem. Physiol. 37:911 (1959).
18. Fiske, C.H., and Y. Subbarow, J. Biol. Chem. 66:375 (1925).
19. Barlett, G.R., Ibid. 234:466 (1959).
20. Dole, V., J. Clin. Invest. 35:150 (1956).
21. Mahadevan, S., C.J. Dillard and A.L. Tappel, Analyt. Biochem. 27:387 (1969).
22. Skipski, V.P., and M. Barclay, "Methods in Enzymology," Vol. XIV, Academic Press, New York, 1966, p. 566.
23. Appleton, H.D., B.N. La Du, Jr., B.B. Levy, J.M. Steele and B.B. Brodie, J. Biol. Chem. 205:803 (1953).
24. Ottolenghi, A., and F. Bernheim, Rad. Res. 15:609 (1961).
25. Dawson, R.M.C., N. Hemington and J.B. Davenport, Biochem. J. 84:497 (1962).
26. Fleischer, S., and B. Fleischer, "Methods in Enzymology," Vol. X, Academic Press, New York, 1967, p. 427.
27. Wood, W.A., "Methods in Enzymology," Vol. II, Edited by S.T. Colowick and N.O. Kaplan, Academic Press, New York, 1955, p. 214.
28. Kaback, H.R., Ann. Rev. Biochem. 39:561 (1970).
29. Kaback, H.R., E.M. Barnes, Jr., and L.S. Milner, in "Recent Advances in Microbiology," Edited by A. Perez-Miravete and D. Pelaez, Liberia Internacional, S.A., Mexico City, 1970, p. 171.
30. Kaback, H.R., "Current Topics in Membranes and Transport," Vol. I, Edited by A. Kleinzeller and F. Bronner, Academic Press, New York, 1970, p. 35.
31. Kaback, H.R., "The Molecular Basis of Membrane Transport," Edited by D. Tosteson, Prentice-Hall, Inc., Englewood Cliffs, N.J., 1969, p. 421.
32. Fleischer, S., A. Casu and B. Fleischer, Fed. Proc. 23:2305 (1964).
33. Koike, W., K. Iida and W. Watscio, J. Bacteriol. 97:448 (1969).
34. Bernheim, F., J. Gen. Microbiol. 34:327 (1964).
35. Bernheim, F., Microbios. 1A:23 (1969).
36. Bernheim, F., Ibid. 4:87 (1971).
37. Wallach, D.F.H., J. Gen. Physiol. 54:115 (1969).
38. Elsbach, P., J.W.O. Van Den Berg, H. Van Den Bosch and L.L.M. Van Deenen, Biochem. Biophys. Acta 106:338 (1965).
39. Elsbach, P., J. Goldman and P. Patriarca, Ibid. 280:33 (1972).
40. Weglicki, W.B., M. Waite, P. Sisson and S.B. Shohet, Ibid. 231:512 (1971).
41. Bangham, A.D., and R.M.C. Dawson, Ibid. 59:103 (1962).
42. Bonsen, P.P.M., G.H. deHaas, W.A. Pieterson and L.L.M. Van Deenen, Ibid. 270:364 (1972).
43. Lankisch, P.G., L. Lege, H.D. Oldigs and W. Vogt, Ibid. 239:267 (1971).
44. Lankisch, P.G., and W. Vogt, Ibid. 270:241 (1972).
45. Roelofsen, B., R.F.A. Zwaal, P. Comfurius, C.B. Woodward and L.L.M. Van Deenen, Ibid. 241:925 (1971).
46. Patriarca, P., S. Beckerdite, P. Pettis and P. Elsbach, Ibid. 280:45 (1972).
47. Simpkins, H., E. Panko and S. Tay, J. Membrane Biol. 5:334 (1971).
48. Abbot, N.J., T. Deguchi, D.T. Frazier, K. Murayama, T. Narahashi, A. Ottolenghi and C.M. Wang, J. Physiol. 220:73 (1972).

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SHORT COMMUNICATIONS

High Phospholipase Content of Intestines of Mice Infected with *Hymenolepis nana*

ABSTRACT

Infection with the tapeworm *Hymenolepis nana* results in the presence of very high amounts of phospholipase in the small intestine of the mouse. The time course and location of this response and its changes following treatment with anti-inflammatory and antiparasitic agents indicate that the enzyme accumulation is part of the reaction of the host to parasitic invasion.

The experiments reported here originated from the observation of unusually high levels of phospholipase activity in the intestines of some mice supplied by a local breeding farm. Considerable variations in enzyme concentration from animal to animal were immediately apparent and could not be obviated by changing diet or time of feeding. An initial tentative correlation between high phospholipase content and worm infection was finally proven by infecting parasite-free mice with *Hymenolepis nana* (*H. nana*) eggs. The following is an account of some experimental observations on the time course, localization and intensity of the enzyme accumulation.

Except for the initial observations on locally supplied mice, Swiss albino mice (Charles River CD-1) were used in all experiments. The animals were housed in groups of 20 per cage in a room with controlled temperature, humidity and light cycle (14 hr light, 10 hr darkness). As a rule the mice were allowed a 5 day rest between arrival and administration of the *H. nana* eggs. The details of the procedure for isolation of the parasites from a colony of infected mice, the preparation of egg suspensions and their administration are given in an accompanying paper (1). The mice were sacrificed at selected time intervals after infection and the accumulation of phospholipase activity was measured by estimation of the lysophospholipase content of homogenates of small intestine, according to the technique already

TABLE I

Lysophospholipase Distribution in Small Intestine of Mice Infected with *Hymenolepis nana*

Day[a]	Intestinal segment[b] I	II	III	IV	Stage of worm development[c]
1	198	132	127	631	Penetration of villi of upper half of intestine by onchospheres
2	201	140	210	779	Development of suckers and hooks and growth of cysticercoids
3	225	237	237	887	
4	425	415	362	850	Growth of cysticercoids is complete
5	1083	3850	550	1366	Migration of cysticercoids begins
6	2306	2200	1133	9400	Migration to lower half of intestine is complete
8	1000	1467	5000	78,733	Attachment and growth in distal segments
11	745	1340	11,383	35,650	
14	8350	24,450	22,750	58,400	Eggs in feces

[a]Time elapsed from egg administration.

[b]Consecutive segments of small intestine from duodenal (I) to cecal (IV) valve. Enzymatic activity is expressed as micromoles of lysolecithin hydrolyzed per gram of tissue per hour. Numerical data are the average of four experimental animals except for days 5, 6 and 8 when three mice were examined.

[c]Condensed from Hunninen (2).

described (1).

In the first series of experiments the temporal relationship between infection and intestinal phospholipase activity was examined. The pooled data from many groups of mice are summarized in Figure 1, where the lysophospholipase activity per gram of small intestine is expressed in logarithmic units to condense the wide range of values found experimentally. As shown, the enzyme concentration began to rise 5 days after infection, was at peak values between the 20th and the 40th days when the parasites are most numerous, and remained at rather high values up to the 81st day, which is our last test date. In addition to the evidence presented in Figure 1, the high enzyme level at days 20-40 has been confirmed in more than 200 mice used over the past 2 years for the preparation and biochemical characterization of the mouse intestinal phospholipase (1). Only a few infected animals (5 out of 100) failed to respond, while control uninfected mice housed in our facilities up to 40 days after arrival consistently exhibited very low activities (average 900 units/g/hr, none above 2000 units).

The relationship between parasite development and accumulation of phospholipase was also studied by determining the distribution of the enzyme in different sections of the small intestine during the initial period of the infection. For this purpose the small intestine was cut in four segments of equal length to be tested separately. The results are given in Table I, together with a brief chronology of the various phases of development of the tapeworm as described by Hunninen (2). It appears that the rise in enzyme concentration is synchronous with the migration of the newly emerging adult form from the proximal segments (days 4-5) to the distal part of the intestine, where

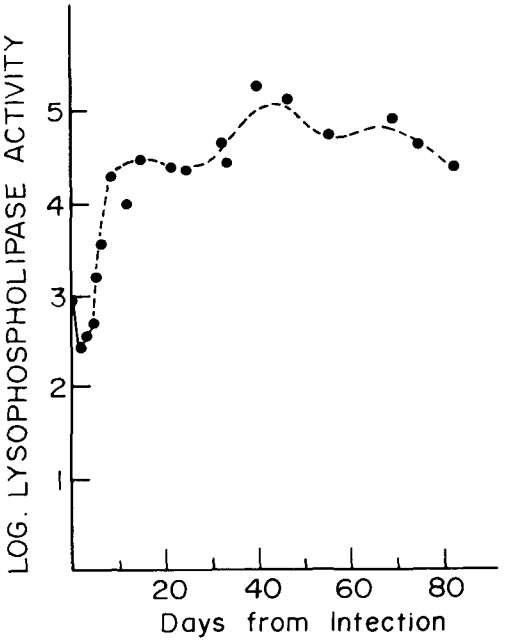

FIG. 1. Lysophospholipase activity of intestines of worm-infected mice. On the ordinate: hydrolytic rates (micromoles of substrate hydrolyzed per gram of tissue per hour) expressed in logarithmic units. Each point is average of three experimental animals, except for days 0-4 and 11, 14, 32 when four or more mice were examined.

the fully developed form is most often located. It is noteworthy that at later times all segments exhibit a progressive rise in enzymatic activity, suggesting a generalized and sustained reaction affecting all the intestinal tissue.

Administration of an antiinflammatory steroid (Dexamethazone) drastically reduced the phospholipase concentration of the intestine of infected mice to levels only one order of

TABLE II

Effect of Dexamethazone on Lysophospholipase Content of Mouse Intestine[a]

Experimental group		
Control, uninfected	Infected untreated[b]	Infected, treated[c]
571	75,000	3700
210	70,000	3700
1000	53,000	6800
631	45,000	9400

[a]Lysophospholipase activity expressed as micromoles of lysolecithin hydrolyzed per gram of tissue per hour.

[b]1000 eggs orally on day 0; four animals examined on day 21.

[c]1000 eggs on day 0, dexamethazone (8 mg/kg ip) on days 20 and 21; four animals examined on day 22.

TABLE III

Effect of Niclosamide on Intestinal Lysophospholipase of Mice Infected with *Hymenolepis nana*[a]

Experimental group		
Infected, untreated	Day 4 of treatment	Day 8 of treatment
40,400	8600	2836
45,000	3600	8262
24,000	6940	2792
91,000	—	1802

[a]All animals were infected with 5000 eggs orally. Beginning on day 18, seven mice were administered niclosamide orally (500 mg/kg in aqueous suspension) twice a day for 3 days (6 doses). The enzymatic content of the intestine was determined on days 21 and 25. Two infected, untreated mice were examined on each sampling day.

magnitude above those of control mice (Table II).

Equally effective was the administration of niclosamide, a vermifuge active against tapeworms (Table III).

Homogenates of *H. nana* eggs or parasites at various stages of development were uniformly negative with regard to lysophospholipase activity. Application to mouse intestine of a histochemical method for phospholipase B previously developed for rat intestine (3) showed a strong positive reaction of the entire length of the intestine of infected animals. Control mice were only faintly positive.

These findings establish unequivocally a causal relationship between worm infestation and increased intestinal phospholipase activity. While the precise meaning of this reaction of the host organism remains to be assessed, a new and unsuspected role for the enzyme is indicated.

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REFERENCES

1. Ottolenghi, A., Lipids 8:415 (1973).
2. Hunninen, A.V., Amer. J. Hyg. 22:414 (1935).
3. Ottolenghi, A., J.P. Pickett and W.B. Greene, J. Histochem. Cytochem. 14:907 (1966).

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Lowering of Serum Cholesterol by Intestinal Bacteria in Cholesterol-Fed Piglets

INTRODUCTION

Higher serum cholesterol concentrations have been reported in germ-free (1) or antibiotic-treated animals on high cholesterol diets than in conventional animals raised on the same diets.

It has been suggested that when germ-free animals develop an intestinal microflora, microbial conversion of cholesterol to coprostanol and of primary bile acids to secondary bile acids is responsible for a concomitant decrease in serum cholesterol levels, an increase in neutral sterol excretion and an increase in bile acid turnover rates (2,3). The difficulty in testing that hypothesis has been that in addition to alteration of intestinal sterol composition, bacteria also may alter intestinal transit time, change the morphology and absorption capabilities of the intestine, and increase the bulk of the feces—factors that may also affect serum cholesterol concentration. In the present experiment, conditions were found that increased the numbers of intestinal bacteria and altered sterol metabolism but did not change the kinds of fecal sterols found in the gnotobiotic animals.

EXPERIMENTAL PROCEDURES

As part of another study (4) in this laboratory, four hysterectomy-derived Duroc pigs were raised in 3 x 4 x 3 ft stainless steel, germ-free isolators on a sterilized 0.5% (dry weight) cholesterol (HC) milk formula (Table I).

At 2-3 weeks of age, the animals were monocontaminated with *L. acidophilus*, since our experience has shown that when swine are moved from germ-free conditions to a nonsterile environment this monocontamination gives them a much better chance of survival. *L. acidophilus* was used because it is normally found in milk-fed animals and has no known capacity to metabolize bile acids and cholesterol (5). At the end of 6 weeks total feces were collected for 5 days. Two pigs were then placed in an isolator well with the plastic top opened. These animals were allowed to develop an intestinal flora in specific pathogen-free facilities for 3 weeks on unsterilized HC milk formula diet, while the other two pigs remained in the isolator on the sterile HC milk formula. At 9 weeks of age the total feces were again collected from each group for 5 days.

The total number of fecal organisms was determined at 6 and 9 weeks in Gall's anaerobic medium and tryptose broth as reported previously (6).

Steroid Analysis

Total serum cholesterol and fecal neutral steroids were determined at 6 and 9 weeks of

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TABLE I

Composition of Pig Diet

Component	High cholesterol milk, 2 liters
Whole milk	1565 ml
Egg yolks[a]	8-10
Cream	40 g
Casein hydrolysate	56 g
Lactose	14 g
Vitamin solution[b]	10 ml
Mineral mix[c]	10 ml
β-Propiolactone-H_2O 6:54 v/v	60 ml

[a]Egg yolk was added to give a 0.5% cholesterol content (dry weight).

[b]Vitamin mix per liter contained: 200 g choline chloride, 40 g inositol, 40 mg biotin, 20 mg folic acid, 1.0 mg vitamin B_{12} (1.0 g of 0.1%), 600 mg riboflavin, 160 mg thiamine, 1 g pantothenic acid, 1.6 g niacinamide, 120 mg vitamin B_6, 16 g vitamin A (4 x 10^6 IU), 0.4 g vitamin D_3 (7 x 10^4 IU), 1.6 g vitamin E (DL α-tocopherol acetate, 400 IU), 20 g vitamin C (ascorbic acid).

[c]Mineral mix per liter contained: 4.0 g $MgSO_4 \cdot 7H_2O$, 3.8 g $MnCl_2 \cdot 4H_2O$, 0.26 g KI, 3.9 g $CuSO_4 \cdot 5H_2O$, 49.8 g $FeSO_4 \cdot 7H_2O$.

age by gas liquid chromatography (GLC). Serum was saponified and extracted according to Abell et al. (7). Tridecanoin was added as an internal standard for GLC analysis. An aliquot of the solution was injected on a 1/4 in. x 3 ft glass column packed with 3% OV-17 on 100-120 mesh Gas Chrom Q (Applied Science Lab.) at 300 C.

As commonly found in germ-free animals, the feces were semiliquid and were washed from the isolators with small additional portions of water. The feces were thoroughly homogenized in a large Waring blender and 5.0 or 10.0 ml aliquots were taken in duplicate for steroid analysis. Next 30,000 dpm of 24-^{14}C-deoxycholic acid (5.72 mC/mM) was added to each sample as a recovery standard. The feces were dried, saponified and the neutral sterols extracted with hexane according to Evrard and Janssen (8). Fecal neutral steroids were determined by GLC as described above. The saponification mixture was acidified and the acidic steroids extracted with diethyl ether. An aliquot was taken and washed through activiated charcoal to remove pigments. An aliquot of the clarified extract was evaporated in a counting vial and radioactivity was determined by liquid scintillation spectroscopy. Recovery of the radioactivity was always 95-100%. The remainder of the extract was evaporated to dryness and a GLC standard of 50-300 μg of 5α-cholanic acid 3β-ol was added. Bile acid methyl esters and trifluoroacetate derivatives were prepared and analyzed by GLC as described previously (9).

RESULTS

At 6 weeks of age the pigs weighed ca. 30 lb and the two groups were ingesting identical amounts of cholesterol by 9 weeks—1360 mg/day.

In spite of a large increase in numbers of fecal bacteria in the animals moved to the nonsterile environment, there was no change in the composition of fecal neutral steroids or ratio of hyocholic to hyodeoxycholic acids (Table II). However, in these animals a dramatic decrease in serum cholesterol was accompanied by a four-fold excretion of neutral steroids compared to pigs that remained in the sterile isolator on the same diet. Over 99% of the neutral fecal steroids in both groups, as determined by GLC, appeared to be cholesterol. No plant sterols were detected since the formula was derived from animal sources only.

Bile acid excretion by the animals in the nonsterile environment was lower than by the isolator group. Although this is contrary to results obtained from rats (10), it seems reasonable that depletion of the cholesterol pool would result in decreased bile acid synthesis and excretion.

The results indicate that factors other than microbial metabolism of steroids in the gastrointestinal tract are responsible for the alteration in serum cholesterol and neutral steroid excretion under these conditions.

DISCUSSION

Most studies of the effects of intestinal

TABLE II

Relationship between Intestinal Flora, Fecal Steroids and Serum Cholesterol in Swine

Environment	Age, weeks	Serum cholesterol, mg/100 ml	Fecal steroids				Total aerobes/g feces	Total anaerobes/g feces
			Total excretion, mg/day		Bile acid composition, %			
			Neutral sterols	Bile acids	HCA[a]	HDC[a]		
ii[b]	6	1. 646	132	279	61	3.9	1.4×10^7	—
		2. 766	(8.8 mg/g feces)					
		3. 564						
		4. 657						
ii	9	1. 447	159	617	63	2.8	4.8×10^7	1×10^8
		4. 421	(5.1 mg/g feces)					
oi[b]	9	2. 216	684	413	77	2.8	9.5×10^7	1×10^{10}
		3. 124	(21.4 mg/g feces)					

[a]HCA = hyocholic acid; HDC = hyodeoxycholic acid.
[b]*ii* = remaining in isolators; *oi* = removed from isolators at 6 weeks of age.

bacteria on overall regulation of steroid metabolism have concentrated on microbial alterations of fecal steroid composition. Other effects of bacteria have been largely ignored.

The morphology of the germ-free intestine is quite different from that of the conventional animal (11,12), and absorption of intact protein and unhydrolyzed triglyceride (13) is possible. The effect on cholesterol absorption is not known.

Other factors such as bulk of the feces, intestinal transit time and binding of bile acids by intestinal bacteria, any of which could alter sterol balance, are also influenced by the intestinal bacteria. Also, Kellogg and Wostmann (10) have suggested that mucosal sloughing could contribute to the greater excretion of neutral steroids by conventional than by germ-free rats.

The present work and that of Kellogg (14) indicate that other factors may be more important than microbial steroid metabolism in explaining the observed differences in steroid flux in gnotobiotic and conventional animals.

Several of the above possibilities which could be readily studied, such as cholesterol absorption, altered intestinal transit time, differences in intestinal structure and careful examination of the role of various bacteria, should be the object of future work on this problem.

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REFERENCES

1. Danielson, H., and B. Gustafsson, Arch. Biochem. Biophys. 83:482(1959).
2. Beher, W.T., G.D. Baker, W.L. Anthony and D.G. Penny, Proc. Soc. Exp. Biol. Med. 116:442 (1964).
3. Wostmann, B.S., N.L. Wiech and E. Kung, J. Lipid Res. 7:77 (1966).

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4. Reiser, R., Circulation 44:2, II-3 9(1971).
5. Aries, V., J. Crowther, B.S. Draser and M.J. Hill, Gut 10:575 (1969).
6. Graber, C.D., R.W. Moore, M. Suzuki, W.E. Redmond, R.M. O'Neil and B. Lockhart, J.Bacteriol. 92:1290 (1966).
7. Abell, L.L., B.B. Levy, B.B. Brodie and F. Kendall, J. Biol. Chem. 195:357 (1952).
8. Evrard, E., and G. Janssen, J. Lipid Res. 9:226 (1968).
9. Mott, G.E., R.W. Moore and R. Reiser, Ibid. 12:117 (1971).
10. Kellogg, T., and B. Wostmann, Ibid. 10:495 (1969).
11. Dubos, R.J., D.C. Savage and R.W. Schaedler, Dis. Colon Rectum 10:23 (1967).
12. Thompson, G.R., and P.C. Trexler, Gut 12:230 (1971).
13. Hahn, P., and O. Koldovsky, "Utilization of Nutrients during Postnatal Development," Pergamon Press, New York, 1966, p. 89.
14. Kellogg, T.F., Fed. Proc. 30(6):1808 (1971).

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Extraction of Prostaglandins E_1, E_2 and E_3 from Human Seminal Plasma

ABSTRACT

Isolation of individual prostaglandins of the E group from human seminal plasma consists of three main steps: preliminary purification from other lipids; resolution of this partially purified material into various groups of prostaglandins on a column of silicic acid; and further purification and resolution of various prostaglandins of each group by reversed phase partition chromatography. The last step is time-consuming and has been replaced by a two-step thin layer chromatography: first on silica gel, which ensures purification of the PGEs, followed by argentation thin layer chromatography, which resolves them into E_1, E_2 and E_3 in microgram quantities.

Human seminal plasma consists of thirteen different prostaglandins (PGs), which are grouped as prostaglandins E, F, A and B, and 19-OH derivatives of A and B. There are three members in each of the E and F groups, the members of which are called primary prostaglandins because they are not interconvertible in biological systems (1). Hamberg and Samuelsson (2) have described a procedure for the isolation of individual prostaglandins from seminal plasma at pH 3.0 as a crude mixture that also consists of neutral and polar lipids. This mixture is further resolved into different groups on a silicic acid column. From each group the individual prostaglandins are resolved by reversed phase partition chromatography. For the localization of the individual members of the E group, an aliquot of each chromatographic fraction has to be examined for the presence of the PGs by means of the rise in the extinction at 278 nm in the presence of alkali. This is due to the formation of a conjugated di-enone.

Instead of this time consuming process, the present report concerns a modified procedure that avoids the reversed phase chromatographic step. Thus a fast thin layer chromatographic (TLC) step on Silica Gel G followed by TLC on silver nitrate-treated silica gel may be used for the isolation, in pure state, of microgram quantities of prostaglandin E compounds.

MATERIALS AND METHODS

Human seminal plasma was obtained from the Copenhagen Health Insurance Organization. It was stored in ethanol 1:3 v/v at -20 C. The PGE markers used were isolated from seminal plasma by the standard method of Samuelsson (2,3). PGE_1 was obtained from Unilever, Ltd., Vlaardingen, The Netherlands.

Preliminary purification of the PGs present in 300 ml seminal fluid, followed by their resolution on silicic acid column, was done according to Hamberg and Samuelsson (2) and Samuelsson (3). The ethylacetate-benzene eluate 6:4 v/v from the silicic acid column was concentrated by distilling off the solvent under reduced pressure (10 mm Hg). The residue containing 9.87 mg of PGs (Table I) was quantitatively taken up in 10 ml chloroform and 200 μl from this solution was used for further purification and quantitation on each of five plates of Silica Gel G (0.4 mm), using the solvent ethylacetate-isooctane-acetic acid-water 110:20:10:100 v/v mixture equilibrated for 1 hr before using the organic phase (4). The zone due to the PGE compounds (Rf 0.49) was scraped off and eluted three times with 4 ml of methanol. The extracts from the first two plates were used for the quantitation of the total content of the PGE compounds by measuring the chromophore at 278 nm in the

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7. Abell, L.L., B.B. Levy, B.B. Brodie and F. Kendall, J. Biol. Chem. 195:357 (1952).
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Instead of this time consuming process, the present report concerns a modified procedure that avoids the reversed phase chromatographic step. Thus a fast thin layer chromatographic (TLC) step on Silica Gel G followed by TLC on silver nitrate-treated silica gel may be used for the isolation, in pure state, of microgram quantities of prostaglandin E compounds.

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TABLE I

Resolution of PGE Compounds by Argentation Thin Layer Chromatography

Volume of seminal fluid used	PGE compounds in ethyl-acetate-benzene 6:4 v/v eluate	PGE[a] compounds (197 μg) resolved by argentation TLC from 200 μl of the 10 ml $CHCl_3$ solution		
	Dissolved in 10 ml $CHCl_3$	E_1	E_2	E_3
300 ml	9.870 mg	47.19(μg) (23.90%) 44.48(μg) (22.53%) 50.36(μg) (25.51%)	38.41(μg) (19.46%) 41.05(μg) (20.79%) 43.10(μg) (21.83%)	Variable from 13.8 μg to 25.7 μg (7 % to 13 %).

[a]Figures indicate the absolute quantities (μg) of the PGE compounds. The figures within parentheses refer to the respective percentages calculated on the basis of the total PGE content in the ethylacetate-benzene eluate.

presence of 0.5 M sodium hydroxide (2). The methanolic extracts from the last three plates were used for the argentation TLC (2).

The prostaglandins were recovered by scraping off the silica gel, transferring it into tubes, emulsifying with water, and acidifying the emulsion with acetic acid to pH 3.0, followed by extraction with an equal volume of ethylacetate three times. The mixed ethylacetate phase was washed with a minimum amount of water until neutral and evaporated to dryness under N_2. The residues were dissolved in methanol and assayed as described above.

RESULTS AND DISCUSSION

In Table I, the absolute amounts of PGE_1, PGE_2 and PGE_3 isolated by argentation TLC and the yields calculated on the basis of total prostaglandin content of the ethylacetate-benzene eluate are indicated.

The ethylacetate-benzene eluate 6:4 v/v of the silicic acid column was found to contain impurities which were more polar, as well as less polar, than the prostaglandin E compounds (5). However, the PGEs were freed from such impurities in one step of TLC separation. This became evident from the fact that only three zones corresponding to the three prostaglandins E_1, E_2 and E_3 were revealed in the subsequent argentation TLC. The three zones were identified as E_1, E_2 and E_3, respectively, by comparison of the Rf values with those of the standard markers (E_1, Rf = 0.74; E_2, Rf = 0.54; and E_3, Rf = 0.35). However a zone close to the solvent front was also revealed. A spectrophotometric examination of the material present in this zone revealed, in the presence of alkali, a slight but significant (10% of the PGE_1) ΔE_{278} value, which could be due to some degradation-metabolite of the PGE compounds.

For biological assay of PGs, removal of silver ions is necessary and this can be done by exposing the chromatograms to H_2S (6). However this is not advisable since it results in a loss of biological activity, due to the corresponding release of HNO_3. Kunze and Bohn (7) have demonstrated that an almost total destruction of PG (E_1, E_2, $F_{1\alpha}$ and $F_{2\alpha}$) activity takes place in a 5N solution of HNO_3, HCl, H_2SO_4 and $HClO_4$. An acidity corresponding to 5N HNO_3 is obtained in plates containing 0.2 g $AgNO_3$ per gram silica gel when these plates are exposed to H_2S gas. The losses in the biological activity may be due to low pH (8). Horton and Main (9) have suggested that removal of silver ions could also be achieved by treatment with sodium chloride solution, thus converting silver nitrate to insoluble AgCl. The extraction procedure used here does not cause the formation of

HNO_3. Acetic acid is commonly used for the acidification of mixtures containing prostaglandins prior to their extraction with organic solvents; no loss in the biological activity takes place with this acid. Thus the separated PGs could well be used for biological assay, either dissolved in saline or Tyrode solution (7).

Most authors have used argentation TLC for establishing the purity of PGE compounds and not for their quantitative assay. There is, however, one report by Kunze and Bohn (7), who have employed $AgNO_3$-impregnated silica gel layers for extractions of microgram quantities of PGE_1, PGE_2, $PGF_{1\alpha}$ and $PGF_{2\alpha}$ as free acids prior to their biological assay. These authors have reported recovery yields of 80-100% using 0.1-10 μg quantities of PGs from up to 0.2 g $AgNO_3$ per gram of silica gel. In our method, the average recovery of the total PGE compounds in the first TLC separation is 98.5% whereas the recovery from $AgNO_3$ TLC was ca. 60% (PGE_1, 22.5-25.5%; PGE_2, 19.4-21.8%; and PGE_3, 7-13% of the total PGE content in the 197.5 μg sample [Table I]). The discrepancy between the recoveries in the method of Kunze and Bohn and our method seems to be due to the fact that they used 0.1-10 μg prostaglandins and a biological assay, which is more sensitive than the spectrophotometric one. Further, they might have applied pure prostaglandins on the $AgNO_3$ TLC plate as reference compounds and actually not have resolved them from their mixture. In such a case they could scrape a generous area of the silica gel because there would be no need to worry about cross contamination. In our own experiments with pure PGE compounds, at least 80% of 5-10 μg quantities applied as spots on the plate was recovered after $AgNO_3$ TLC. In the present report, we have used ca. 200 μg of the PGE compounds as a mixture. The three PGE compounds were revealed as zones, which were marked quickly on the plate because, as the time passes and water dries up from the plate, the zones broaden and ultimately lose their identity. In order to recover pure PGE compounds, a conservative area corresponding to each prostaglandin E compound on the plate was marked and eluted; 20-25% of the total PGE compounds could be recovered from the excluded regions. By taking this loss into account, we estimate that the recovery of PGE's from the mixture was similar to those obtained using single reference compounds (ca. 80%). Although the recoveries are not quantitative, the present technique provides a simple and rapid method for the separation of individual PGE compounds.

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REFERENCES

1. Bergström, S., "Proceedings of the Second Nobel Symposium, Stockholm, 1966, Edited by S. Bergström and B. Samuelsson, Alquist and Wicksell, Stockholm, 1967, p. 25.
2. Hamberg, M., and B. Samuelsson, J. Biol. Chem. 241:257 (1966).
3. Samuelsson, B., Ibid. 238:3229 (1963).
4. Bygdeman, M., K. Svanborg and B. Samuelsson, Clin. Chim. Acta 26:373 (1969).
5. Clausen, J., and K.C. Srivastava, Lipids 7:415 (1972).
6. Suzuki, T., and W. Vogt, Naunyn-Schmiedebergs, Arch. Exp. Path. Pharmak. 252:68 (1965).
7. Kunze, H., and E. Bohn, Nauyn-Schmiedebergs, Arch. Pharmak. 267:380 (1970).
8. Von Euler, U.S., J. Physiol. (London) 88:213 (1936).
9. Horton, E.W., and I.H.M. Main, Brit. J. Pharmacol. 30:582 (1967).

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Net Increase of Lipid Fatty Acid in Ehrlich Ascites Carcinoma during Growth

ABSTRACT

The net rate of total lipid fatty acid accumulation was measured in cells and extracellular fluid of Ehrlich ascites carcinomas grown *in* mice. Between 5 and 12 days after ip tumor inoculation, the tumor volume increased from 2.5 to 17.7 ml and the tumor cell volume increased from 1.5 to 3.9 ml. Total lipid fatty acids increased from 60 to 160 μmol during this same period. Thus the amount of fatty acid present increased at a rate of ca. 10 nmol/min for the tumor (cells and

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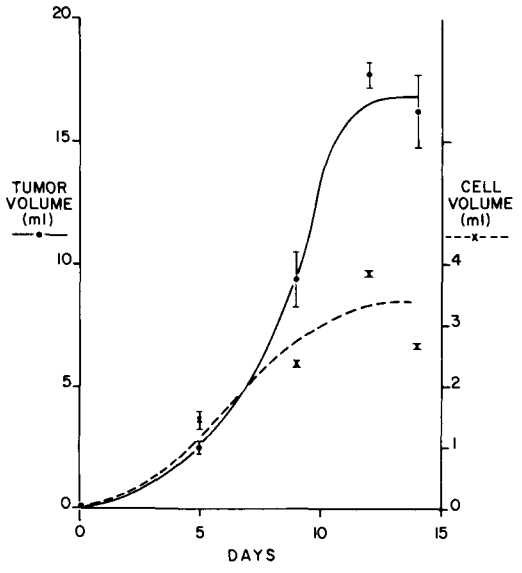

FIG. 1. Growth of Ehrlich ascites tumor in Swiss Webster mice following ip injection of 1.7 x 10^7 washed tumor cells.

fluid). This rate is much smaller than the rate of fatty acid influx into Ehrlich ascites tumor cells in vivo. The major lipid fatty acids in both whole tumor and extracellular fluid were 16:0, 18:0, 18:1 and 18:2; no major changes in fatty acid composition were observed during growth.

Ehrlich ascites carcinoma cells are capable of rapid fatty acid (FA) uptake and metabolism in vitro (1,2) and in vivo (3). Spector (3) has evaluated the significance of free fatty acids (FFA) in tumor nutrition and concluded that the uptake of FA by Ehrlich ascites carcinoma cells in vivo is almost exactly equivalent to that needed for net growth. Spector's conclusion was based upon the assumption that large tumors (>10 ml) had a doubling time of 24 hr. However Klein and Révész (4) have reported that, for large tumors, the time required for the tumor cells to double in number or volume was ca. 10 times as great as the value used by Spector. Thus it appeared to us that the uptake of FFA by tumor cells (3) may be very much greater than the net rate of FA accumulation due to tumor growth. In order to explore this very crucial aspect of tumor lipid metabolism, and as a quantitative basis for our own future studies, we have measured the net rate of lipid fatty acid accumulation in Ehrlich ascites carcinomas.

Ehrlich ascites carcinomas were grown ip in male, Swiss Webster mice (Hilltop Lab Animals, Inc., Los Angeles). The mice were housed at 21 C (no light from 4 P.M. to 8 A.M.) and were fed Purina laboratory chow ad libitum. To initiate tumor growth, tumor cells were harvested from the peritoneal cavity after 7 days growth, washed in Krebs-Henseleit bicarbonate buffer (5), resuspended in buffer, and injected ip (1.7 x 10^7 cells in 0.20 ml). At varying stages of tumor growth, the mice were killed by cervical fracture and the tumor was collected rapidly from the peritoneal cavity into a graduated centrifuge tube maintained at 4 C. Tumor and packed cell volumes were measured. On the 5th and 12th days of growth, 1.0 ml of tumor was removed and transferred within a few seconds into a glass-stoppered tube containing an internal standard, pentadecanoic acid and 10% KOH in 50% methanol. Tumor extracellular fluid was obtained by dilution and rapid millipore filtration; 0.3 ml of whole tumor was delivered into 4.7 ml cold Krebs-Ringer bicarbonate buffer and filtered within 5 sec through a 0.45 μ, 25 mm diameter millipore filter attached to a 10 ml plastic syringe into tubes containing the internal standard and methanolic KOH. All samples were gassed with nitrogen and saponified at 60 C for 16 hr. The nonsaponified lipids were extracted with petroleum ether and discarded. The samples, containing saponified FA, were acidified and the FA extracted with petroleum ether (bp range 30-60 C). The solvent was evaporated under nitrogen prior to methylation with BF_3/MeOH

TABLE I

Fatty Acid Content and Composition of Ehrlich Ascites Carcinoma during Growth

Tumor, days	Tumor lipid fatty acid		Relative fatty acid composition, %						
	μmol/ml	μmol/whole tumor	14:0	16:0	16:1	18:0	18:1	18:2	20:4
5	24.6 ± 6.5	60	0.76 ±0.17	14.0 ±1.3	1.7 ±0.10	16.7 ±3.4	20.4 ±1.4	38.2 ±3.4	8.3 ±1.5
12	8.9 ± 0.6[a]	160	2.1± ±0.90	16.4 ±1.5	2.5 ±0.55	20.1 ±2.2	18.4 ±1.4	30.5 ±3.9	7.0 ±2.6

[a]All values are mean ± SE of tumors (cells + fluid) from five mice per group, except for value indicated with superscript (n=4).

(6). The fatty acid methyl esters were analyzed using a Packard Gas Chromatograph Model 805 equipped with a U-shaped glass column (6 ft x 1/8 in. ID), packed with 15% Hi Eff 2BP on 100-120 mesh Gas Chrom P (Applied Science Labs., State College, Pa.); temperatures: column, 175 C; flash heater, 195 C; and flame ionization detector, 195 C. Nitrogen was used as a carrier gas (40 ml/min). Peak areas were estimated (peak height x peak width at half height) and related to the area of the pentadecanoic acid standard. From these data one could calculate both total lipid FA (TLFA) content and FA composition. The time interval chosen to evaluate the rate of total lipid FA accumulation was based upon the tumor growth curves shown in Figure 1. The mean rate of lipid accumulation between the 5th and 12th days should approximate the tangent to the curve and should also yield a fair estimate of the rate of lipid increase during the period in which we have studied other quantitative aspects of lipid metabolism in these tumors (Mermier and Baker, in preparation). As shown in Figure 1, the mean tumor volume (cells and fluid) increased from 2.5 to 17.7 ml, and the corresponding, mean packed cell volume increased from 1.5 to 3.9 ml between the 5th and 12th days of tumor growth. The mean fractional cell volumes (packed cells/whole tumor) decreased from 0.58 to 0.22 in this period. The marked decrease in fractional cell volumes in older tumors indicates that the extracellular fluid accumulated faster than the tumor cells grew.

The total lipid FA content of Ehrlich ascites tumors after 5 and 12 days growth is shown in Table I. Although the TLFA concentration decreased from 25 to 9 μmol/ml, the total quantity of FA (TLFA concentration x tumor volume) increased. The extracellular fluid which accumulated during tumor growth contained a much lower concentration of TLFA than did the cells (2.2 ± 0.7 [n = 5] and 1.0 ± 0.2 [n = 5] μmol in the extracellular fluid of 1.0 ml whole tumor at 5 and 12 days, respectively; Table I, column 2). However the FA patterns were very similar to those shown in Table I for whole tumor TLFA. There were no gross changes in the qualitative pattern of lipid FA in the whole tumor during growth. The major fatty acids were 16:0, 18:0, 18:1 and 18:2. The latter was the most prevalent FA; this contrasts sharply with similar studies by Yamakawa et al. who reported only trace amounts of 18:2 in 7-day-old Ehrlich ascites carcinomas (7). However our results agree well with Spector's analysis of cell lipid FA (3). The only major discrepancy was that whereas Spector found equal quantities of 18:1 and 18:2 in the tumor cell lipids, we consistently found more 18:2 than 18:1 in both whole tumor and in the ascites fluid.

Based upon these data, we may estimate the order of magnitude of net lipid FA accumulation between the 5th and 12th days of tumor growth, during the period that the tumor volume (cells and fluid) increased from 2.5 to 18 ml and the packed cell volume increased from 1.5 to 3.9 ml. The total lipid FA accumulation (cells and fluid) in 7 days was ca. 100 μmol. This is an average rate of 10 nmol FA per minute.

We are presently carrying out quantitative studies of FFA flux into Ehrlich ascites carcinoma cells under the same conditions that we have used to measure net FA accumulation in the tumor. These studies indicate that the flux of FA into tumor cells is ca. 26 times greater than the rate of FA accumulation during growth. A similar conclusion may be reached if we use the data published by Spector to estimate FA flux into Ehrlich ascites carcinoma cells in vivo. (We estimate from Spector's data [3] that the flux of fatty acids into tumor cells was about 18 μEq/hr for tumors of 10 ml volume. This is 30-fold greater than the rate at which total lipid fatty acids increased during the present study of tumor growth. Our estimates of FFA flux into tumor cells is based upon the following values [3]: extracellular fluid FFA concentration, 0.18 μEq/ml; rate constant, 0.23/min; 0.75 ml extracellular fluid per milliliter whole tumor; and ca. 100% FFA uptake by cells. The latter value follows from Spector's observation that 40-50% of injected FFA was found in tumor cells in 10 min; this corresponds to the total amount of injected FFA which disappeared from the extracellular fluid in that time.) The significance of these observations will be discussed elsewhere.

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REFERENCES

1. Fillerup, D.L., J.C. Migliore and J.F. Mead, J. Biol. Chem. 233:98 (1958).
2. Spector, A.A., and D. Steinberg, Cancer Res. 27:1587 (1967).
3. Spector, A.A., Ibid. 27:1580 (1967).
4. Klein, G., and L. Révész, J. Nat. Cancer Inst. 14:229 (1953).
5. Umbreit, W.W., R.H. Burris and J.F. Stauffer, in "Manometric Techniques and Tissue Metabolism," Revised edition, Burgess Publishing Co., Minneapolis, Minn., 1957, p. 149.
6. Van Wijngaarden, D., Anal. Chem. 39:848 (1967).
7. Yamakawa, T., N. Ueta and R. Irie, Japan J. Exp. Med. 32:289 (1962).

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Brain Gangliosides of Myelin Synthesis-Deficient Mice

ABSTRACT

Study of the brain gangliosides of normal and myelin synthesis deficient mice shows no appreciable differences in the quantitative or qualitative composition. This result shows that gangliosides have little or no relation to the synthesis of myelin.

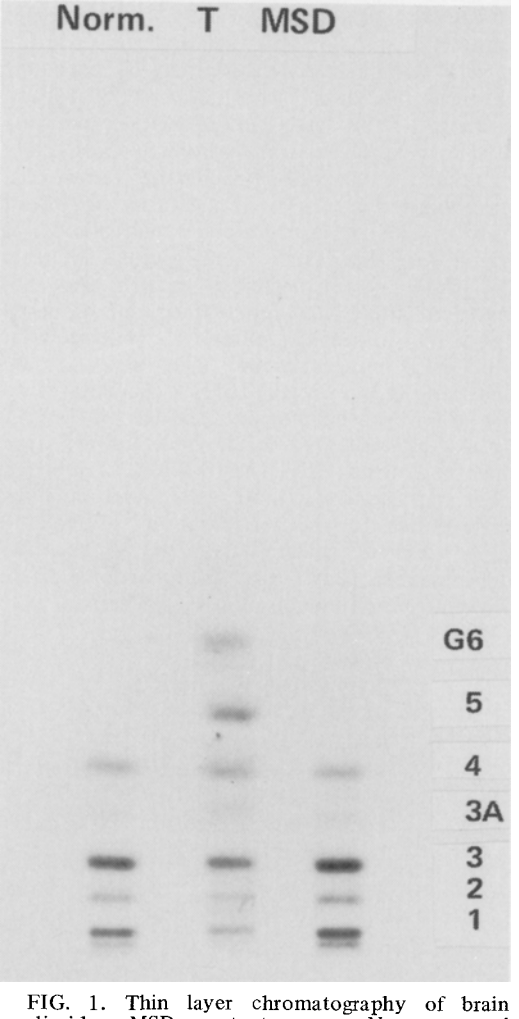

FIG. 1. Thin layer chromatography of brain gangliosides. MSD: mutant mouse; Norm; normal mouse; T: gangliosides obtained from pig brain + G_5 and G_6.

Although changes in the levels and metabolism of cholesterol, fatty acids and some phosphatides have been described, the principal disturbance observed in mice with deficiency in myelin synthesis (Quaking, Jimpy or myelin synthesis-deficient [MSD]) concerns the glycolipids (1-9). Studies with Quaking and Jimpy mice have shown that while the synthesis of the cerebrosides + sulphatides group is notably diminished, the synthesis of gangliosides is only slightly perturbed (10-12). Cerebrosides and sulphatides are also in lower quantity in MSD mice (8). Meier and MacPike (8) have found that the amount of total gangliosides is the same in MSD as in normal mice; however no information on the gangliosides pattern in the brain of such mice has been reported.

Mutant and normal MSD mice were killed at the end of the myelination period (22 days). Gangliosides were obtained from the freeze-dried brain by the method of Suzuki (13). The Folch upper phase was dialyzed against water and freeze-dried. The residue was taken in chloroform-methanol 2:1 v/v and submitted to a mild alkaline hydrolysis modified from Schneider and Kennedy (14). The alkali-stable lipids were then dialyzed and freeze-dried. The final residue was used for neuraminic acid assay and thin layer chromatography (TLC). Neuraminic acid was measured according to Mietinen and Luukainen (15). TLC was performed on silicic acid precoated plates (Merck F254) with chloroform-methanol-water 60:35:8 v/v containing 20 mg $CaCl_2$ per 100 ml solvent (16).

Gangliosides were detected with an orcinol-HCl reagent (17) and quantified by scanning on a Vernon densitometer. Gangliosides were also separated by descending TLC (18). In this case the lipids were determined by the technique of MacMillan and Wherrett (19).

When the amounts of gangliosides per brain are compared, the values obtained (Table I) are quite similar in the two kinds of mice. The difference is notably greater when the results are expressed on the basis of dry or wet weights. This could be related to the fact that following the absence of myelin in the mutant mouse, dry or wet weights are not the best

REFERENCES

1. Fillerup, D.L., J.C. Migliore and J.F. Mead, J. Biol. Chem. 233:98 (1958).
2. Spector, A.A., and D. Steinberg, Cancer Res. 27:1587 (1967).
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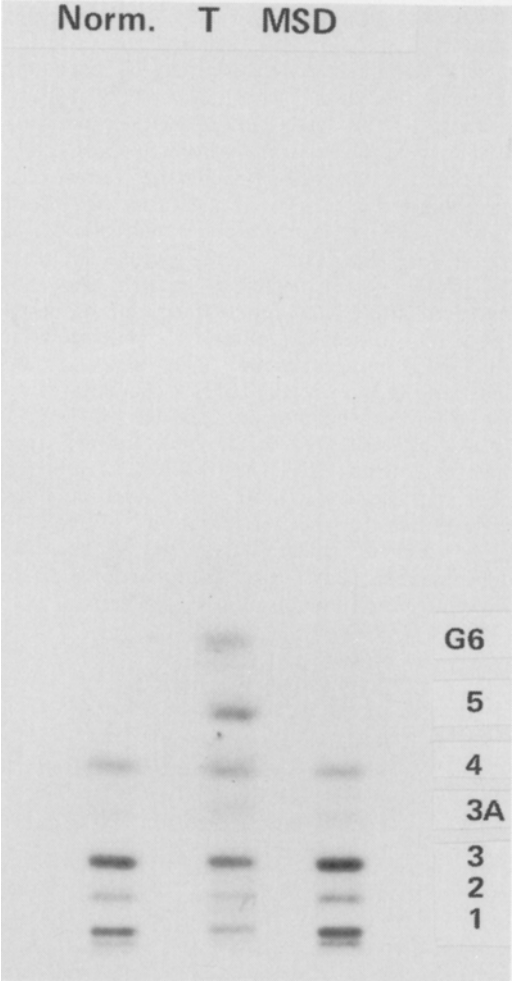

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Mutant and normal MSD mice were killed at the end of the myelination period (22 days). Gangliosides were obtained from the freeze-dried brain by the method of Suzuki (13). The Folch upper phase was dialyzed against water and freeze-dried. The residue was taken in chloroform-methanol 2:1 v/v and submitted to a mild alkaline hydrolysis modified from Schneider and Kennedy (14). The alkali-stable lipids were then dialyzed and freeze-dried. The final residue was used for neuraminic acid assay and thin layer chromatography (TLC). Neuraminic acid was measured according to Mietinen and Luukainen (15). TLC was performed on silicic acid precoated plates (Merck F254) with chloroform-methanol-water 60:35:8 v/v containing 20 mg $CaCl_2$ per 100 ml solvent (16).

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TABLE I

Total Lipid Sialic Acid in Normal and Myelin Synthesis-Deficient Mice Brains

	Normal		Myelin synthesis-deficient
μg NaNa[a]/brain	81.1 ± 9.9		90.5 ± 17.0
μg NaNa/g wet wt	211.5 ± 22.2		257.2 ± 34.6
μg NaNa/100 mg dry wt	110.9 ± 10.0		134.8 ± 32.9
Means μM NaNa/g wet wt			
Our results	0.682		0.832
Values of Meyer and MacPike (8)	0.568	to	0.874

[a]NaNa = N-acetyl neuraminic acid. All values are the mean of five different experiments.

references for a comparison. The amount of gangliosides that we found was in the same range as the values given by Meier and MacPike (8). Since the amount of gangliosides in the MSD mouse is equal to or even higher than that in the normal one, this class of lipids probably is not involved in the striking myelin deficiency found in this strain. Suzuki et al. (20) have reported the existence of gangliosides in purified myelin. However the amount found by these authors is too low to indicate that even a severe myelin deficiency could influence notably the amount of total brain gangliosides.

The gangliosides patterns in the brains of normal and MSD mice (Table II, Fig. 1) are very similar. Our results suggest that as in the Quaking and Jimpy mice (10) there is no great alteration of the metabolic pathway of the glucosyl ceramide. These conclusions agree well with the work of Brenkert et al. (21), who observed no difference in the brain UDP-glucose ceramide glucosyl transferases of MSD and normal mouse brain.

In contrast to rat brain, we have found only one report upon the distribution of gangliosides in mouse brain. The pattern that we found is quite similar to that reported by Kostic et al. (22) on the normal Jimpy strain mice. The major difference is the absence from the MSD strain of the G_{2A} gangliosides which account for 10% of the total Jimpy gangliosides. This is perhaps a strain specificity, but it could also be explained by the great differences in the separative and analytical methods used in the two studies.

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P. Guerin provided technical assistance. W. Reyman manufactured the device with which descending chromatography was performed.

REFERENCES

1. Mandel, P., J.L. Nussbaum, N.M. Neskovic, L.L. Sarliève and T. Kurihara, Adv. Enz. Reg. 10:101 (1972).
2. Kandutsch, A.A., and S.E. Saucier, Biochim. Biophys. Acta 260:26 (1972).
3. Jacque, C.M., M.L. Harpin and N.A. Bauman, Eur. J. Biochem. 11:218 (1969).

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TABLE II

Ganglioside Pattern of Brain[a]

	Normal mice	Myelin synthesis-deficient
GM_1/G_4	12.2 ± 3.3	11.2 ± 2.2
GD_3/G_{3A}	3.7 ± 1.2	3.3 ± 1.5
GD_{1a}/G_3	38.2 ± 6.7	38.4 ± 3.9
GD_{1b}/G_2	14.6 ± 2.4	14.9 ± 1.9
GT_1/G_1	22.2 ± 2.5	22.0 ± 2.6
GQ_1/G_O	10.1 ± 4.2	10.2 ± 1.5

[a]All values are the mean of five different experiments.

4. Singh, H., N. Spritz and B. Geyer, J. Lipid Res. 12:473 (1971).
5. Joseph, K.C., M.J. Druse, L.R. Newell and E.L. Hogan, J. Neurochem. 19:307 (1972).
6. Deshmukh, D.S., T. Inoue and R.A. Pieringer, J. Biol. Chem. 246:5695 (1971).
7. Hauser, G., J. Eichberg and S. Jacobs, Biochem. Biophys. Res. Commun. 43:1072 (1971).
8. Meier, H. and A.D. MacPike, Exp. Brain Res. 10:512 (1970).
9. Reasor, M.J., and J.N. Kanfer, Life Sci. 8:1055 (1969).
10. Neskovic, N.M., L.L. Sarliève and P. Mandel, Brain Res. 42:147 (1972).
11. Sarliève, L.L., N.M. Neskovic, G. Rebel and P. Mandel, Neurobiology 2:70 (1972).
12. Mathieu, J.M., U. Schneider and N. Herschkowitz, Brain Res. 42:433 (1972).
13. Suzuki, K., Life Sci. 3:1227 (1964).
14. Schneider, P.B., and E.P. Kennedy, J. Lipid Res. 9:58 (1968).
15. Miettinen, T., and I.T. Luukainen, Acta Chem. Scand. 13:856 (1959).
16. Van Den Eijnden, D.H., Z. Physiol. Chem. 352:1601 (1971).
17. Klenk, E., and W. Gielen, Z. Physiol. Chem. 326:144 (1961).
18. Korey, S.R., and J. Gonatas, Life Sci. 5:296 (1963).
19. MacMillan, V.H., and J.R. Wherrett, J. Neurochem. 16:1621 (1969).
20. Suzuki, K., S.E. Poduslo and W.T. Norton, Biochim. Biophys. Acta 144:375 (1967).
21. Brenkert, A., R. Arora, N.S. Radin, H. Meier and A.D. MacPike, Brain Res. 36:195 (1972).
22. Kostic, D., J.L. Nussbaum and P. Mandel, Life Sci. 8:1135 (1969).

[Revised manuscript
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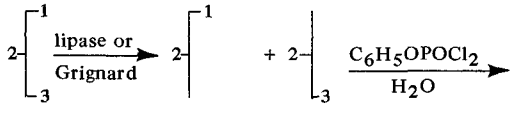
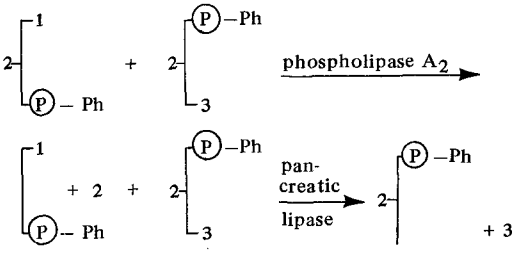
LETTER TO THE EDITOR

Determination of Fatty Acids in Position 3 of Triglycerides

Sir: In the most frequently used method of stereospecific triglyceride analysis, 1,2(2,3)-diglycerides (obtained from triglycerides with pancreatic lipase or by Grignard deacylation) are converted to 1(3)-phosphatidylphenol, of which only the *sn*-3-isomer is hydrolyzed by phospholipase A_2 (Brockerhoff, H., Lipids 6:942 [1971]). The fatty acids in positions 1 and 2 can be directly analyzed, fatty acid 2 in two different products, but the fatty acid composition in 3 must be calculated by difference. Although two independent ways are open for this calculation and the accuracy of the analysis can be judged by the agreement of the results, a method to analyze the fatty acids in 3 directly would still be desirable, not so much for additional confirmation of the analysis but because of the uncertainty involved in the quantitation of minor and trace fatty acids in 3 merely by difference. A direct analysis of position 3 is possible with a method starting from 1,3-diglyceride, but this intermediate cannot be prepared from triglyceride except contaminated with 6-10% isomerized 1,2(2,3)-diglyceride (Brockerhoff, H., Lipids 6:942 [1971]). A different method to analyze position 3 directly is proposed in the following scheme (Ⓟ = phosphate; Ph = phenol).





The conventional stereospecific analysis is followed, but in an additional step the *sn*-1-phospholipid, isolated by thin layer chromatography, is hydrolyzed in position 3 by pancreatic lipase. Slotboom et al. (Chem. Phys. Lipids 4:15 [1970]) have shown that this reaction can proceed to completion. Crude pancreas powder could be used, since the *sn*-1-phospholipid would not be digested by pancreatic phospholipase A_2. Esterification of the free phosphate hydroxyl of the phospholipid, e.g., with diazomethane, would accelerate the final lipolytic reaction.

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ERRATUM

Two errors occurred in the publication of "Absorption and Biliary Secretion of Intraperitoneally Injected 3β-Methoxycholest-5-ene-4-^{14}C in the Rat" by L.N. Norcia (Lipids 8:315[1973]). A line of the first complete paragraph on page 317 and part of the caption to Figure 3 (p. 318) were omitted. Those portions of the paper are printed correctly below.

For purposes of comparison, a few studies of secretion of label into bile following ip injection of cholesterol-4-^{14}C were made. Amounts of cholesterol-4-^{14}C or methoxycholestene-4-^{14}C used for the ip injections were ca. 1 mg of compound of specific activity ca. 10 μCi/mg. The compounds were injected as suspensions in 0.7 ml of 0.9% saline stabilized with 1 drop of Tween 20 (5).

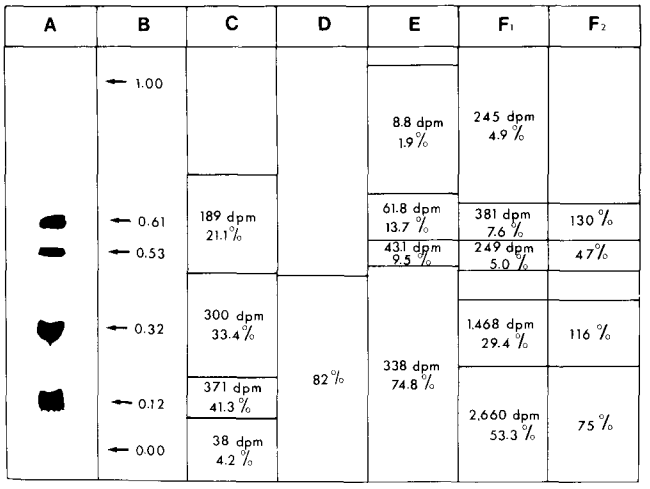

FIG. 3. Methoxycholestene metabolites in bile, studied by thin layer chromatography, experiment 2 (see legend, Fig. 2). Thin layer chromatography on Silica Gel G, 250 μ thick, solvent system toluene-acetic acid-chloroform-water 80:36:20:1 v/v. Diagram is drawn to scale of R_f values of chromatograms. Positions of areas to be scraped off were determined by chromatographing in duplicate in adjacent lanes, then spraying one lane with phosphomolybdic acid spray while protecting adjacent lane with baffle. After color development and spot visualization (sprayed with 5% phosphomolybdic acid in ethanol-diethyl ether 1:1, then heated at 100 C for 3-5 min), areas to be scraped from untreated lane were marked. Panel A: diagram of typical chromatogram of extract of hydrolyzed bile. Panel B: R_f values, methoxycholestene, 0.61; cholesterol, 0.53; chenodeoxycholic acid, 0.32; cholic acid 0.12. Panel C: distribution of radioactive counts from hydrolyzed bile extract, first 24 hr bile sample after ip injection; dpm and % total counts are given for eluate from plate scrapings. Panel D: % polar metabolites from fraction shown which were bound to Amberlite IRA-400-OH resin, first 24 hr bile sample. Panel E: dpm and % total counts, first 24 hr bile sample. Panel F_1: dpm and % total counts, second 24 hr bile sample. Panel F_2: eluates from F_1 recrystallized three times with nonradioactive carrier compounds; % recovery of dpm in crystallized compounds corrected for losses during crystallization.

Fatty Acid Composition of Sarcoplasmic Reticulum Phospholipids from Red and White Muscles of the Rabbit

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ABSTRACT

The fatty acid composition of the phospholipids of sarcoplasmic reticulum preparations from rabbit psoas (white) and soleus (red) muscles was determined. The sarcoplasmic reticulum from psoas muscle was lower in stearic and oleic acids and higher in palmitic and linoleic acids than that from soleus muscle, and contained a greater proportion of polyunsaturated fatty acids. However most of the differences in fatty acids were small.

In skeletal muscle the sarcoplasmic reticulum is responsible for regulation of the calcium ion concentration at the active sites of the contractile proteins. The nature of calcium transport across this membrane has been studied extensively, and its dependence upon membrane lipids is well established (1-4) although the exact role of the lipids is unknown. In addition, comparative studies have shown that sarcoplasmic reticulum preparations from red muscles differ from those from white muscles in their ability to transport calcium ions (5,6). The purpose of the present study was to determine whether or not this difference in the ability to transport calcium ions might be due to a difference in the fatty acid composition of the sarcoplasmic reticulum phospholipids of red and white muscles.

Sarcoplasmic reticulum was isolated from psoas (white) and soleus (red) muscles of seven New Zealand White rabbits as described by Martonosi et al. (7), except that it was washed only once with 0.6 M KCl-buffer and 1 mM dithiothreitol was present in buffers throughout the preparation. Lipids were extracted from the sarcoplasmic reticulum preparations with chloroform-methanol (8), and the phospholipids were separated from the total lipid extract by thin layer chromatography (9). The area containing the phospholipids was scraped from the plate, and the phospholipids were eluted (10). Methyl esters of the phospholipid-fatty acids were prepared by heating the phospholipids (ca. 1 mg) for 16 hr at 65 C with 1 ml of 5% (v/v) sulphuric acid in methanol in sealed ampoules, and the resulting methyl esters were extracted with petroleum ether (bp 30-40 C). The lipid extracts were handled and stored under nitrogen at all times. The fatty acid composition of

TABLE I

Fatty Acid Composition of Sarcoplasmic Reticulum Phospholipids from Rabbit Psoas (White) and Soleus (Red) Muscle[a]

Fatty acid	Psoas (white)	Soleus (red)	SE of difference	Significance of difference
15:1	4.6 ± 0.8	2.2 ± 0.4	± 0.7	< 0.02
16:0	22.0 ± 1.3	16.3 ± 0.9	± 0.8	< 0.001
16:1	1.7 ± 0.2	1.6 ± 0.2	± 0.2	NS[b]
17:1	1.2 ± 0.2	0.8 ± 0.1	± 0.2	NS
18:0	13.9 ± 0.4	21.6 ± 1.1	± 1.3	< 0.01
18:1	19.4 ± 1.2	24.3 ± 0.3	± 1.2	< 0.01
18:2	15.0 ± 1.0	11.7 ± 1.2	± 1.2	< 0.05
20:4	13.6 ± 1.1	10.9 ± 0.9	± 1.7	NS
Unidentified[c]	1.6 ± 0.3	2.8 ± 0.7	± 0.5	< 0.05
22:5	2.0 ± 0.5	2.2 ± 0.5	± 0.3	NS
Total Polyenoic[d]	32.2 ± 1.5	27.7 ± 1.4	± 1.5	< 0.05

[a]Values, expressed as percentages of the total fatty acids, are means ± SE for muscle preparations from seven animals. Each phospholipid preparation was analyzed in duplicate. Fatty acids with relative concentrations below 1% in both muscle preparations are omitted.

[b]NS = not significant.

[c]Relative retention time 3.4 (18:0 = 1.0).

[d]Unidentified acid assumed to be polyenoic.

the methyl esters was determined by gas liquid chromatography using a 10 ft x 1/8 in. column containing 5% EGSS-X on Chromosorb W A/W DMCS 30/100 mesh using a Varian Aerograph gas chromatograph with nitrogen as carrier gas. The column, injection port and flame ionization detector oven were operated at 180, 280 and 245 C, respectively. The percentage of an individual fatty acid methyl ester was determined from the area under the appropriate peak and the area under all peaks. Peaks were tentatively identified by comparison of their retention times with those of standards obtained from Poly Science Corp. Ill. and Supelco, Pa., and in certain cases by separating the total methyl esters into groups according to degree of saturation by argentation thin layer chromatography (11) with subsequent gas liquid chromatography.

Table I shows the fatty acid composition of the phospholipid of sarcoplasmic reticulum isolated from the two different muscles. The preparations from the psoas muscles contained significantly greater proportions of pentadecenoic acid (15:1), palmitic acid (16:0) and linoleic acid (18:2) than those from soleus muscles. On the other hand the soleus phospholipids contained greater proportions of stearic acid (18:0) and oleic acid (18:1). The proportion of polyunsaturated fatty acids in the psoas muscle preparations was significantly greater than that in the soleus muscle preparations.

We have recently shown that the relative proportions of fatty acids of the sarcoplasmic reticulum can be considerably changed without affecting its calcium transporting properties (12,13). In view of the comparatively small differences in composition between the two types of preparation reported here, it seems unlikely that the known differences in calcium transport between red and white muscles can be attributed to differences in the fatty acid composition of their membrane phospholipids.

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REFERENCES

1. Martonosi, A., J.R. Donley, A.G. Pucell and R.A. Halpin, Arch. Biochem. Biophys. 144:529 (1971).
2. Greaser, M.L., R.G. Cassens, W.G. Hoekstra and E.J. Briskey, Biochim. Biophys. Acta 193:73 (1969).
3. Fiehn, W., and W. Hasselbach, Eur. J. Biochem. 9:574 (1969).
4. Fiehn, W., and W. Hasselbach, Ibid. 13:510 (1970).
5. Sreter, F.A., Arch. Biochem. Biophys. 134:25 (1969).
6. Fiehn, W., and J.B. Peter, J. Clin. Invest. 50:570 (1971).
7. Martonosi, A., J. Donley and R.A. Halpin, J. Biol. Chem. 243:61 (1968).
8. Folch, J., M. Lees and G.H. Sloane-Stanley, Ibid. 226:497 (1957).
9. Krell, K., and S.A. Hashim, J. Lipid Res. 4:407 (1963).
10. Arvidson, G.A.E., Ibid. 8:155 (1967).
11. Morris, L.J., in "Metabolism and Physiological Significance of Lipids," Edited by R.M.C. Dawson and D.N. Rhodes, John Wiley & Sons Ltd., London, 1964, p. 641.
12. Newbold, R.P., R.K. Tume and D.J. Horgan, J. Food Sci., In press (1973).
13. Tume, R.K., R.P. Newbold and D.J. Horgan, Arch. Biochem. Biophys. In press (1973).

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AY-9944 Inhibition of Sterol Biosynthesis in *Chlorella emersonii*[1]

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ABSTRACT

When *Chlorella emersonii*, a green alga, was cultured in the presence of 20 ppm AY-9944, a number of sterols accumulated which appear to be intermediates of sterol biosynthesis in this organism. The sterols isolated include 14α-methyl-ergost-8-en-3β-ol, 14α-methyl 24S-stigmast-8-en-3β-ol, 14α-methyl ergosta-8,24(28)-dien-3β-ol and 4α, 14α-dimethyl 24S-stigmast-8-en-3β-ol. Smaller quantities of several other sterols were found in addition to the normally occurring Δ^7-ergostenol, chondrillasterol and Δ^7-chondrillastenol. Control cultures were found to contain, in addition to the normally occurring sterols, smaller quantities of most of the sterols isolated from AY-9944 inhibited cultures. AY-9944 is a specific inhibitor of Δ^7-reductase in cholesterol biosynthesis in animals. However, since *C. emersonii* terminates sterol biosynthesis one step prior to the Δ^7-reductase step, AY-9944 apparently inhibits sterol biosynthesis prior to this step in this organism. The accumulation of 14α-methyl sterols in treated cultures suggests that AY-9944 is an effective inhibitor of the 14α-methyl removal in *C. emersonii*.

INTRODUCTION

The hypocholesterolemic drug *trans*-1,4-bis-(2-chlorobenzylaminomethyl) cyclohexane dihydrochloride (AY-9944) is a well known inhibitor of the reduction of 7-dehydrocholesterol to cholesterol in animals (1-3). Recently AY-9944 has been shown to be an inhibitor of sterol biosynthesis in algae (4,5). However it seems certain that rather than being a Δ^7-reductase inhibitor, AY-9944 is primarily an inhibitor of the reduction of the Δ^{14} double bond in the $\Delta^{8,14}$ sterol biosynthetic intermediates described in these reports.

Previous studies in this laboratory involved the use of *Chlorella ellipsoidea*, a green alga that normally produces only Δ^5 sterols. When such unpredicted intermediates were obtained with this alga, we felt some insight into the mechanism of inhibition by AY-9944 might be gained if the effect of AY-9944 was determined on an organism that does not normally carry out the Δ^7-reductase step at all, viz., an alga synthesizing only Δ^7 sterols. *Chlorella emersonii* is such an organism (6). The effects of AY-9944 on sterol synthesis in *C. emersonii* (if any) must therefore be on steps other than Δ^7-reductase.

EXPERIMENTAL PROCEDURES

Chlorella emersonii var. *emersonii*, Shihira and Krauss, Maryland Culture Collection No. 2 (Indiana Culture Collection No. 252) was cultured axenically in 15-1 carboys on basal inorganic medium supplemented with 0.5% glucose as previously described (5). AY-9944-treated cultures were grown in the same way as the controls except that at the time of inoculation AY-9944 was added to give a final concentration of 20 ppm. Cells grown at this level of AY-9944 produced ca. 50% the yield of control cultures. Sterols were extracted from freeze-dried cells with $CHCl_3/CH_3OH$ 2:1 v/v and after saponification were partially purified by digitonin precipitation as described by Doyle et al. (7).

Further purification and separation was accomplished using alumina and $AgNO_3$-impregnated silica gel and Anasil B column chromatography. Gas liquid chromatography (GLC) was carried out with a Glowall Chromalab Model A-110 gas chromatograph . These methods have been previously described (4,7).

RESULTS AND DISCUSSION

Both quality and quantity of sterols were markedly altered when *C. emersonii* was grown in the presence of 20 ppm AY-9944. Total sterol extracted from control and treated cultures was 2.3 mg/g and 1.7 mg/g dry weight, respectively. Relative retention times (RRT) of sterols from control cultures indicate that the major sterols are identical to those described by Patterson (6). They are thus identified as

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TABLE I

Quantitative Comparison of Sterols from Control and AY-9944-Treated Cultures of *Chlorella emersonii*

Sterols	Control		AY-9944-Treated	
	% of sample	μg/g dry wt	% of sample	μg/g dry wt
24-Methylene cycloartanol	0.3	6	tr[a]	tr
24-Dihydroobtusifoliol	0.8	19	0.2	2
4α, 14α-Dimethyl 24S-Stigmast-8-en-3β-ol	0.4	8	0.3	4
14α-Methyl-ergost-8-en-3β-ol	0.9	22	3.4	57
14α-Methyl 24S stigmast-8-en-3β-ol	0.4	8	9.8	165
14α-Methyl-ergosta-8,24(28)-dien-3β-ol	0.8	19	3.6	62
Obtusifoliol	0.2	5	tr	tr
Cycloeucalenol	0.0	0	tr	tr
Δ^7-Ergostenol	16.3	390	42.6	720
Δ^7-Chondrillastenol	8.6	200	9.8	165
Chondrillasterol	70.8	1650	29.2	500
5α-Ergosta-7,22-dien-3β-ol	0.5	12	0.4	7
Total	100.0	2339	99.3	1682

[a]tr = trace; indicates less than 1 μg/g dry wt.

Δ^7-ergostenol, chondrillasterol and Δ^7-chondrillastenol (Table I).

Obvious qualitative and quantitative differences in the initial gas chromatograms (on SE-30 columns) of treated cultures include the presence of five major peaks instead of three, the presence of one completely new major peak, a large increase in the Δ^7-ergostenol peak or a concommitent decrease in the chondrillasterol peak.

Alumina column chromatography separated the sterols into 4,4′-dimethyl, 4-methyl and 4-desmethyl fractions. Following conversion of these fractions into the acetates, the sterols were further separated and purified on $AgNO_3$-impregnated silica gel columns. Sterols thus separated were sufficiently pure for an accurate determination of the RRT of each sterol acetate on three GLC systems (3% SE-30, 1% QF-1, 3% HiEff-8BP) as previously described (5).

Positive identification of these sterols is based on comparative RRT data of free sterols and acetates with published values (8) and with authentic compounds. Identification was based on the characteristic movement of the compounds on alumina and $AgNO_3$-silica gel columns.

A quantitative comparison of control and treated sterols is presented in Table I. A more thorough check of sterols from control cultures revealed the presence of small amounts of many of the sterols found in the treated samples. Significant quantitative changes in sterols of the treated samples are as follows: production of the three major sterols was reduced from 96 to 82% of the total sterol; chondrillasterol was reduced by the greatest amount (1650 to 500 μg/g dry wt,) while Δ^7-ergostenol nearly doubled (390 to 720 μg/g dry wt); and there were large increases—as great as 20-fold—in the 14α-methyl Δ^8 sterols.

Obviously AY-9944 has a pronounced effect on sterol biosynthesis in *C. emersonii*. Accordingly AY-9944 must be more than a Δ^7-reductase inhibitor in this organism.

The point or points of this inhibition are not clear from the data; however the following possibilities are postulated. Although 20 ppm of AY-9944 was required in this study and only 5 ppm of triparanol was used by Doyle et al. (7), the result of AY-9944 inhibition is nearly identical to triparanol inhibition in *C. emersonii*. Considering the relatively large accumulation of 14α-methyl sterols (17.3% of total sterol in treated cultures, 3.8% in control cultures), the inhibitory activity of AY-9944 may be similar to that postulated for triparanol, i.e., inhibition of 14α-methyl removal.

The ratio of nine carbon to ten carbon side chains (0.25 and 1.0, in control and treated cells, respectively) is interesting. This is due in large measure to the great increase in Δ^7-ergostenol. This clearly provides the possibility that AY-9944 may be inhibiting the second alkylation reaction in the side chain. This large buildup of Δ^7-ergostenol otherwise cannot be explained. The increase in 24-methylene com-

pounds from 1.3% of total sterol (control) to 3.6% (AY-9944-treated) also suggests that AY-9944 may inhibit the second alkylation reaction.

In conclusion, when these data are compared with data previously reported (4,5) from AY-9944-treated *C. ellipoidea*, i.e., a large accumulation of $\Delta^{8,14}$ sterol dienes and lesser amounts of $\Delta^{8(9)}$ monoenes, the primary sites of inhibition by AY-9944 in sterol biosynthesis of *C. ellipsoidea* are postulated to be at steps 2 or 3 in the sequence shown below:

$$14\alpha\text{-methyl}\Delta^{8(9)}\text{sterols} \xrightarrow{1} \Delta^{8,14}\text{sterol dienes} \xrightarrow{2} \Delta^{8(9)}\text{ sterols} \xrightarrow{3} \Delta^{7}\text{ sterols}$$

However, in *C. emersonii*, at a much higher concentration of inhibitor (20 ppm vs. 4 ppm), inhibition is seen only at site 1 and at the second alkylation in the side chain. Side chain alkylation was not inhibited in *C. ellipsoidea* at 4 ppm AY-9944.

The differential qualitative and quantitative effect observed in accumulated sterols between these two organisms in response to AY-9944 is difficult to explain. That *C. emersonii* required five times the concentration of inhibitor to achieve the same level of growth inhibition as *C. ellipsoidea* indicates that the Δ^{14}-reductase of *C. emersonii* is relatively insensitive to AY-9944. Only at the higher level are the 14α-methyl removal and the second alkylation inhibited. The absence of any identifiable $\Delta^{8(9),14}$ intermediates in *C. emersonii* offers a second more exciting possibility—perhaps *C. emersonii* does not carry biosynthesis through this route at all.

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REFERENCES

1. Dempsey, M., Ann. New York Acad. Sci. 148:631 (1968).
2. Dvornik, D., M. Kraml and J. Bagli, J. Amer. Chem. Soc. 86:2739 (1964).
3. Kraml, M., J. Bagli and D. Dvornik, Biochem. Biophys. Res. Comm. 15:455 (1964).
4. Dickson, L.G., G.W. Patterson, C.F. Cohen and S.R. Dutky, Phytochem. 11:3473 (1972).
5. Dickson, L.G., and G.W. Patterson, Lipids 7:635 (1972).
6. Patterson, G.W., Plant Physiol. 42:1457 (1967).
7. Doyle, P.J., G.W. Patterson, S.R. Dutky and M.J. Thompson, Phytochemistry 11:1951 (1972).
8. Patterson, G.W., Anal. Chem. 43:1165 (1971).

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Acetyl Carnitine Formation in Rat Heart[1]

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ABSTRACT

The comparative incorporation of acetate into long chain fatty acids and acetyl carnitine by cell-free preparations of rat heart has been investigated. Whereas the addition of 1 mM carnitine stimulated (45%) fatty acid synthesis by liver preparations in citrate-containing media, fatty acid synthesis from acetate in rat heart homogenates under the same incubation conditions was markedly depressed. This depression by carnitine of acetate incorporation into long chain fatty acids in 105,000 x g soluble fractions of heart was associated with increased acetyl carnitine formation. Thus in heart tissue acetyl CoA is effectively shuttled into acetyl carnitine and is unavailable for synthesis of fatty acids. These data are in agreement with results obtained earlier in studies with perfused rat heart. A similar conversion of added acetyl CoA to the carnitine derivative occurred when labeled malonyl CoA was used as fatty acid precursor, again resulting in reduced fatty acid synthesis. It was shown by direct measurement that acetyl carnitine formation in the absence of carnitine was greatest in heart mitochondria and least in microsomes. In the presence of carnitine, acetyl carnitine formation was increased in all subcellular fractions, with the greatest change again occurring with mitochondria.

INTRODUCTION

Friedman and Fraenkel (1) first reported the enzymatic acetylation of carnitine by liver extracts, and concluded that the ester bond of acetyl carnitine is energetically comparable to the thioester bond of acetyl CoA. Several suggestions have since been made as to the metabolic function of the enzyme, carnitine acetyl transferase (CAT), and of acetyl carnitine itself. Studies from several laboratories (2-6) have been consonant with the hypothesis that CAT may be involved in transport of acetyl groups out of mitochondria, and more recently it has been suggested that CAT, which has been reported to be present only in mitochondria (7), may be involved in intramitochondrial transfer of acetyl groups between the compartments of β-oxidation and of the tricarboxylic acid cycle (8-11). Pearson and Tubbs (12) have suggested that acetyl carnitine might represent a store of "active" acetyl groups and that its formation releases free CoA for other cellular functions.

In a previous report from this laboratory (13), it was found that carnitine addition to perfusates of isolated rat hearts did not affect the uptake or oxidation of labeled acetate but decreased acetate incorporation into long chain fatty acids. Thus it appeared that there might be another metabolic fate of the acetate taken up by heart muscle under these conditions. In the present study it has been shown that, under incubation conditions designed to study hepatic fatty acid synthesis, acetate conversion to acetyl carnitine by rat heart preparations is markedly stimulated by addition of carnitine. This results in a decrease in acetate incorporation into long chain fatty acids and is in contrast to data obtained using liver preparations as a reference tissue, in which carnitine stimulated fatty acid synthesis from acetate.

EXPERIMENTAL PROCEDURES

Materials

Sodium acetate-1-^{14}C (40 mc/mmol), sodium acetate-^{3}H (40 mc/mmol) and palmitic acid-1-^{14}C (55 mc/mmol) were obtained from Amersham/Searle Corp. Acetyl-1-^{14}C-coenzyme A (54 mc/mmol) was purchased from New England Nuclear Corp., malonyl-1,3-^{14}C-coenzyme A (9.4 mc/mmol) from International Chemical and Nuclear Corp., and carnitine-methyl-^{14}C HCl (11.1 mc/mmol) from Tracerlab. Unlabeled acetyl CoA and malonyl CoA were purchased from Pabst Labs., and other chemicals were obtained from Calbiochem and Sigma.

Preparation of Cell Fractions

Male albino rats (Microbiological Associates Inc.), weighing 250-300 g, were given standard laboratory chow ad libitum until use. The animals were sacrificed by decapitation; hearts and livers were quickly removed, blotted, weighed and placed in 5 volumes 0.24 M sucrose, pH 7.4, containing 0.001 M EDTA. The tissue was homogenized at 0-2 C with a Teflon pestle and glass homogenizing tube, and the crude homogenate was centrifuged at 1000

x g for 15 min in a Sorvall refrigerated centrifuge. Mitochondria were prepared by centrifugation of the 1000 x g supernatant at 8500 x g for 15 min. The pellet was washed with sucrose, recentrifuged at 8500 x g for 15 min, and again resuspended in 0.24 M sucrose.

The supernatant from the 8500 x g centrifugation was centrifuged at 105,000 x g for 1 hr in a Spinco Model L-2 ultracentrifuge, and the resulting microsomal pellet was resuspended in sucrose and centrifuged as before. Protein was determined on all cell fractions by the method of Lowry et al. (14).

Incubation Conditions

The incubation media was a modification of that described by Abraham et al. (15) for studies of fatty acid synthesis, and contained the following components per 2 ml medium: 240 μmol glycyl glycine, pH 7.4; 70 μmol $MgCl_2$, 1 μmol $MnCl_2$; 75 μmol potassium citrate; 50 μmol ATP (K+); 0.2 μmol CoA, 1 μmol NADP+; 1 μmol NADPH; 60 μmol glutathione; 10 μmol $KHCO_3$ and 150 μmol potassium phosphate, pH 7.4. In certain experiments, the following components were also added: 6.2 μmol potassium acetate-1-^{14}C (2 x 10^6 dpm), 1 μmol acetyl-1-^{14}C-CoA (1 x 10^6 dpm); or 1 μmol malonyl-1,3-^{14}C-CoA (1 x 10^6 dpm). The incubation media were placed in 25 ml Ehrlenmeyer flasks, and either 0.1 ml distilled water controls or 0.1 ml *d,l*-carnitine was added to give a final carnitine concentration of 1 mM. The appropriate cell fraction (1.5 ml) was added to initiate the reaction, and the flasks were incubated for 2 hr at 37 C, with shaking.

Analysis of Lipids

The entire incubation mixture was extracted in 20 volumes chloroform-methanol 2:1 v/v according to Folch et al. (16). The chloroform phase was evaporated to dryness under nitrogen and the residual lipid redissolved in 1-5 ml hexane. Aliquots of the hexane extract were placed in scintillation vials, evaporated to dryness under nitrogen and counted by liquid scintillation spectrometry (13). Lipids in the hexane extract were separated into major classes by thin layer silicic acid chromatography in a solvent system of hexane-diethyl ether-acetic acid 88:10:3 v/v. The areas corresponding to authentic phospholipids, monoglycerides, cholesterol, unesterified fatty acids, triglycerides, fatty acid methyl esters and cholesterol esters were individually scraped into scintillation vials. One milliliter methanol and 10 ml scintillant were added prior to counting. All isotope data were corrected to dpm by the channels ratio method (17).

Determination of Acetyl Carnitine

The formation of labeled acetyl carnitine in reaction mixtures was determined as follows: Following incubations of tissue fractions with combinations of acetate-1-^{14}C and carnitine, or with acetate-^{3}H and carnitine-methyl-^{14}C HCl, the entire reaction mixture was extracted in chloroform-methanol (16). The upper aqueous-methanol layer was removed, evaporated to dryness under nitrogen, and the residue was taken up in 0.5 ml of absolute ethanol containing 10 mg carrier acetyl carnitine. Acetyl carnitine was crystallized by addition of 15 ml acetone-diethyl ether 1:2 v/v according to previously described procedures (5,18). The acetyl carnitine was then recrystallized twice from absolute ethanol, dissolved in 3 ml absolute ethanol, and aliquots taken for thin layer chromatography in a solvent system of 4% benzene in methanol. The silicic acid areas corresponding to authentic acetate, carnitine and acetyl carnitine were scraped into scintillation vials, and 1 ml methanol and 10 ml liquid scintillant were added for isotope analysis.

Calculations

Data are expressed in nmoles acetate or malonate incorporated into long chain fatty acids or into acetyl carnitine. Calculation of the incorporation of malonyl-1,3-^{14}C-CoA into fatty acids was based on utilization of one-half of the isotope in the substrate. All differences were analyzed for significance by the student *t* test (19), and the data are expressed as means ± standard error.

RESULTS

The effect of carnitine on incorporation of acetate-1-^{14}C by 8500 x g supernatant fraction of heart into total lipids is shown in Figure 1. Liver was used as a reference tissue, since it has been reported that in this tissue carnitine stimulates fatty acid synthesis from acetate (20). Carnitine concentrations of 0.1 mM and 1.0 mM depressed acetate incorporation into cardiac lipids by 10-38%, while with liver carnitine stimulated acetate incorporation by 40-45% as had been shown previously (20). At the 10 mM concentration, carnitine depressed acetate incorporation into long chain fatty acids in preparations of both tissues. The inhibition of acetate incorporation in heart tissue by carnitine was due to decreased acetate incorporation into the free fatty acid fraction (decrease of 0.87 nmol/mg protein) and the diglyceride fraction (decrease of 0.11 nmol/mg

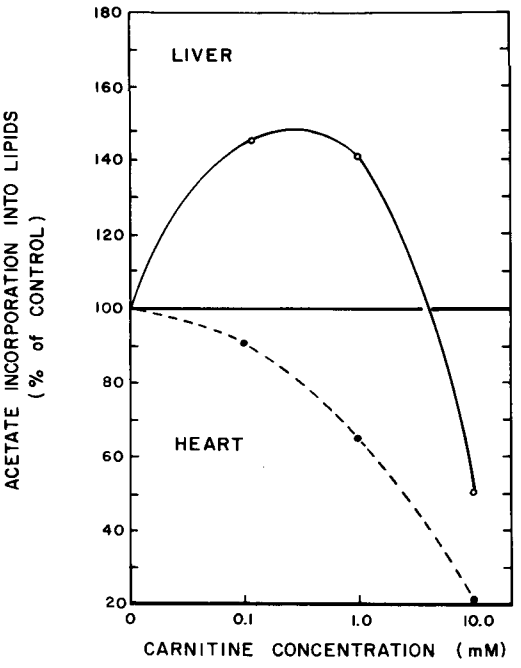

FIG. 1. Effect of carnitine on acetate-1-^{14}C incorporation into lipids by rat liver and heart 8500 x g soluble fractions. Incubation media contained following components in total volume of 3.6 ml: 6.2 μmol potassium acetate-1-^{14}C (1 μCi); 240 μmol glycyl glycine, pH 7.4; 70 μmol $MgCl_2$; 1 μmol $MnCl_2$; 75 μmol potassium citrate; 50 μmol ATP (K+); 0.2 μmol CoA; 1 μmol NADP; 1 μmol NADPH; 60 μmol glutathione; 10 μmol $KHCO_3$; 150 μmol potassium phosphate, pH 7.4; varying concentrations of carnitine; and ca. 8 mg 8500 x g soluble fraction protein. Incubations were carried out for 2 hr at 37 C, and the media were extracted according to Folch et al. (16).

protein), while in liver carnitine stimulation of acetate incorporation into lipids was due almost entirely to increased radioactivity in free fatty acids (increase of 0.53 nmol/mg protein).

In order to determine the effect of 1 mM carnitine on synthesis of fatty acids by various subcellular fractions, three experiments were carried out in which the incorporation of acetate-1-^{14}C and malonyl-1,3-^{14}C were compared in heart homogenates and in mitochondrial, microsomal and soluble fractions. Representative data are shown in Table I and are the means from triplicate incubations ± SEM. When acetate was used as substrate for fatty acid synthesis, the highest total and specific activity (nmoles acetate incorporated per milligram protein) was found in the 105,000 x g soluble fraction. The presence of 1 mM carnitine resulted in a marked depression in acetate incorporation into fatty acids in this fraction as well as in the microsomal fraction. Analysis of the lipid extracts of the incubation medium following acetate incorporation studies showed that, in the 105,000 x g soluble fraction, 81% of the isotope was associated with the free fatty acid fraction on thin layer chromatograms. Following incubations of the 105,000 x g soluble fraction with labeled acetate and carnitine, only 11.6% of the label was in long chain free fatty acids, while 71% remained at the origin on thin layer chromatograms and was determined to be acetyl carnitine.

When labeled malonyl CoA was used as substrate for fatty acid synthesis, total incorporation was again highest in the soluble fraction, while the highest specific activity was in the microsomal fraction. Carnitine (1 mM) de-

TABLE I

Effect of Carnitine (1 mM) on Incorporation of Acetate and Malonyl CoA into Lipids in Heart Cell Fractions

Substrates[a]	Substrate incorporated, nmol		
	Mitochondria	Microsomes	Cytoplasm
Acetate-1-^{14}C	6.8 ±0.6	1.3 ± 0.5	52.1 ± 0.3
Acetate-1-^{14}C + carnitine, 1 mM	5.5 ±1.3	0.3[b] ± 0.2	19.3[b] ± 1.1
Protein, mg	2.1	1.0	8.4
Malonyl-1,3-^{14}C-CoA	7.8 ±6.2	15.8 ± 2.6	48.6 ± 0.8
Malonyl-1,3-^{14}C-CoA + carnitine, 1 mM	5.8 ±5.1	11.4 ± 2.2	21.7[b] ± 3.4
Protein, mg	1.6	0.9	9.0

[a]The composition of incubation medium and conditions are given in Figure 1 and the text. Acetate-1-^{14}C was included at a concentration of 1.72 mM; malonyl-1,3-^{14}C-CoA was included at a concentration of 0.28 mM, and these incubations also contained "sparker" (0.1 mM) acetyl-CoA.

[b]Probability (p)<0.01.

pressed malonyl CoA incorporation into fatty acids to 39% of control in heart homogenates and to 43% of control in the soluble fraction of heart, but was without effect on the mitochondrial and microsomal fractions. Analysis of the lipid extracts of 105,000 x g soluble fraction indicated that in the absence of carnitine, 69% of the label was associated with the free fatty acid fraction on thin layer chromatograms; when carnitine was present in the incubation, only 35% of the label in the lipid extracts was associated with the free fatty acid fraction. The radioactivity remaining at the origin after thin layer chromatography was increased from 21% in the absence of carnitine to 40% when carnitine was present in the media. This radioactivity was shown to be acetyl carnitine following rechromatography of this fraction.

Since long chain acyl carnitines also remain at the origin with phospholipids during silicic acid chromatography of major lipid classes, this fraction was scraped into tubes and extracted with chloroform-methanol 2:1 v/v. Radioactivity was determined on a portion of the extract, and the remainder was subjected to two dimensional thin layer chromatography, according to Wittels and Bressler (21). By this procedure, acyl carnitines are separated from tissue phospholipids. Under the conditions of these studies, there was no formation of long chain acyl carnitines formed during studies on incorporation of acetate or malonate into long chain fatty acids.

Formation of Acetyl Carnitine

After incubations of tritium-labeled acetate and carbon-labeled carnitine with 105,000 x g soluble fraction, the medium was extracted with butanol, and the resulting aqueous phase, along with authentic standards of acetate, carnitine and acetyl carnitine, was subjected to thin layer chromatography in a solvent system of 4% benzene in methanol. The plate was separated into 1 cm sections, and each section was scraped into scintillation vials and counted. A significant portion of the tritium label from acetate was associated with the silicic acid area corresponding to authentic acetyl-^{3}H carnitine (Fig. 2).

Further evidence for the formation of acetyl carnitine was obtained using the procedures of Bremer (18) and Bressler and Katz (5). Authentic acetyl carnitine (10 mg) was added as carrier to the aqueous-methanol phase after chloroform-methanol extraction of the incubation media. The acetyl carnitine was recrystallized, dissolved in absolute ethanol, and an aliquot was counted. Another aliquot was subjected to

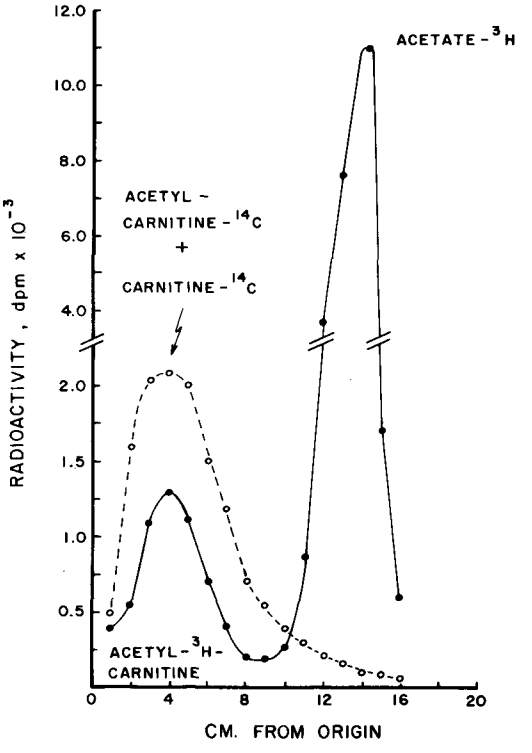

FIG. 2. Thin layer chromatographic separation of free acetate-^{3}H and acetyl-^{3}H carnitine-^{14}C. Reaction mixture contained 210 μmol glycyl glycine, pH 7.4; 70 μmol $MgCl_2$; 1 μmol $MnCl_2$; 75 μmol potassium citrate; 50 μmol ATP (K+); 0.2 μmol CoA; 1 μmol TPN; 60 μmol glutathione; 10 μmol $KHCO_3$; and 150 μmol potassium phosphate, pH 7.4. Acetate-1-^{3}H was included at a level of 6.2 μmol and carnitine-methyl-^{14}C was present at a concentration of 1 mM. The total volume of the medium was 2.1 ml. After addition of 1.5 ml heart 105,000 x g soluble fraction, incubation was carried out for 2 hr at 37 C. Entire incubation mixture was extracted in butanol-water 2:1 and aliquots of remaining aqueous phase were spotted on Silica Gel G thin layer plates. Plates were developed in 4% benzene in methanol and 1 cm sections of silicic acid layer scraped into scintillation vials for counting.

thin layer chromatography in 4% benzene in methanol to determine the extent of free acetate contamination of the recrystallized acetyl carnitine. The data in Table II show that, after 2 hr incubation of the assay medium containing acetate-^{3}H but no enzyme source, only 180 dpm was crystallized with carrier acetyl carnitine, and this was shown by chromatography to be free acetate. The addition of 1 mM carnitine to the medium lacking enzyme did not increase in the amount of radioactivity crystallized with acetyl carnitine. Incubations with either 900 x g supernatant fraction of heart homogenates or the 105,000 x g soluble fraction resulted in a marked incorporation of radioactive acetate into a product coprecipi-

TABLE II

Crystallization of Acetyl-1-^{14}C Carnitine following Incubations of Acetate-1-^{14}C with Heart Homogenates and High Speed Soluble Fraction

Additions to incubation medium[a]	Carnitine, 1 mM	Acetyl-1-^{14}C carnitine crystallized
		dpm
None	–	180
	+	190
Heart homogenate	–	960
	+	11,850
Heart 105,000 x g	–	3,060
soluble fraction	+	17,700
Heart boiled soluble fraction	+	200

[a]See Table I.

tating with carrier acetyl carnitine; thin layer chromatographic analysis of these samples indicated 75-90% of this radioactivity to be acetyl-^{3}H carnitine and the remainder free acetate-^{3}H. The addition of 1 mM carnitine to incubations containing either heart homogenates or 105,000 x g soluble fraction resulted in a further 6 to 12-fold increase in the radioactive material cocrystallized with acetyl carnitine, and of this 70-75% was acetyl-^{3}H carnitine.

Table III is a summary of three experiments on the formation of acetyl carnitine by cytoplasmic, mitochondrial and microsomal fractions of heart homogenates. The data were obtained after a 2 hr incubation in media containing acetate-1-^{14}C, followed by extraction, cocrystallization with carrier acetyl carnitine and thin layer chromatography. While the mitochondrial fraction had the highest activity per milligram protein, smaller amounts of acetyl carnitine were also formed by the microsomal and 105,000 x g soluble fractions. The addition of 1 mM carnitine resulted in marked increases in acetyl carnitine formation by all three subcellular fractions ranging from a 6-fold increase in the mitochondrial fraction to an 11-fold increase in the 105,000 x g soluble fraction.

DISCUSSION

In an earlier report from this laboratory (13), perfusion of rat hearts with 0.5 mM carnitine did not affect myocardial extraction of circulating acetate nor alter the rate or extent of acetate oxidation to CO_2; however carnitine depressed incorporation of acetate into extractable lipids, specifically tissue fatty acids and triglyceride. These data were in contrast to the results with liver, in which it was reported that carnitine stimulates fatty acid synthesis from acetate (20) and from glucose or pyruvate (5).

Pearson and Tubbs (12) have reported that perfusion of rat hearts with 4 mM acetate resulted in a marked decrease in the cellular concentration of free carnitine, which suggested that increased formation of acetyl carnitine occurred during acetate perfusion. This would reduce the free acetate or acetyl CoA concentration, and result in a decrease in acetate incorporation into long chain fatty acids, as noted earlier (13).

It has been reported (22) that with liver preparations fatty acid synthesis from acetate proceeds at a low rate in the absence of citrate. Furthermore addition of carnitine (1 mM) causes a decrease in these low levels of lipogenesis. In the presence of 1 mM citrate, which activates acetyl CoA carboxylase, carnitine stimulates fatty acid synthesis from acetate. It was suggested that in the absence of citrate acetyl CoA was converted to acetyl carnitine in the presence of carnitine, while after citrate activation of acetyl CoA carboxylase carnitine might effectively form palmityl carnitine from the newly synthesized fatty acid. This latter "shuttle" would remove an inhibitor of acetyl CoA carboxylase, namely, palmityl CoA, and simultaneously provide an activator, palmityl carnitine.

In the present studies, citrate was included in all incubation media, and carnitine did in

TABLE III

Formation of Acetyl Carnitine by Heart Subcellular Fractions

Additions to incubation media[a]	Acetyl carnitine formation, nmol/mg protein		
	Cytoplasm	Mitochondria	Microsomes
Acetate, 6.2 μmol	1.2 ± 0.9	4.5 ± 0.2	0.4 ± 0.1
Acetate + carnitine, 3.6 μmol	13.0 ± 3.5[b]	28.8 ± 1.5[c]	3.7 ± 0.7[c]

[a]Basic incubation media, incubation conditions and crystallization of acetyl carnitine are described in Figure 1 and the text. Data are means from three experiments ± SEM.

[b]$p<0.05$.

[c]$p<0.01$.

fact stimulate fatty acid synthesis by cell-free liver preparations, as reported earlier (22). However with heart preparations the primary fate of substrate acetate in the presence of carnitine was the formation of acetyl carnitine, which effectively decreased acetyl CoA concentrations and resulted in a depression of acetate incorporation into fatty acids (Tables I and III). These data with cell-free preparations provide direct support of data obtained by perfusion of intact rat hearts, in which carnitine depressed acetate incorporation into long chain fatty acids (13).

Increased conversion of acetyl CoA to acetyl carnitine, when carnitine was added to heart 100,000 x g soluble fraction, also resulted in a decreased incorporation of malonate carbons into long chain fatty acids (Table I). It is possible that, during preparation of heart subcellular fractions, enzymes of β-oxidation of fatty acids had been released from mitochondria. Thus stimulation of acetyl carnitine formation by carnitine, from either acetate directly or malonate after decarboxylation, might explain decreased fatty acid synthesis in 105,000 x g supernatants of heart homogenates. This latter explanation cannot wholly account for the observed effects of carnitine for the following reasons: (a) The highest levels of incorporation of both acetate and malonyl CoA into long chain fatty acids were in the 105,000 x g soluble fraction; these levels were comparable, and the effect of carnitine on incorporation of both substrates was almost identical. (b) Mitochondria from these preparations oxidize albumin-complexed palmitic acid efficiently, and this is dependent on added carnitine. (c) In a separate study, the incorporation of acetate-1-^{14}C into long chain fatty acids by 105,000 x g soluble fraction was reduced to 75% ± 2% of control in the absence of bicarbonate in the medium and to 73% ± 1.5% of control in the presence of 300 μg avidin. On the other hand, the absence of bicarbonate or the addition of avidin had no significant effect on mitochondrial incorporation of acetate-1-^{14}C into long chain fatty acids. These data suggest that at least a portion of fatty acid synthesis in 105,000 x g soluble fraction of heart is dependent on acetyl CoA carboxylase in the soluble fraction.

It is difficult to reconcile the present findings on carnitine-stimulated acetyl carnitine formation in subcellular fractions of heart with reports that carnitine acetyl transferase is wholly associated with the mitochondrial fraction (7,23,24) and probably bound to the inner mitochondrial membrane (23). However in the present study markers were not used in determining the "purity" of the membranous fractions.

Although the role(s) of acetyl carnitine remains to be fully elucidated, the distribution of acetyl carnitine transferase among various organs appears to be directly related to the magnitude of the carnitine-stimulated increase in fatty acid oxidation (25). It has also been reported that the ratio of acetyl carnitine to acetyl CoA is high in heart (20:1) and low in liver (2:1), which varies inversely with the ability of these tissues to synthesize fatty acids (12). In heart, when acetyl CoA formation is high, as occurs during fatty acid oxidation, it is possible that increased formation of acetyl carnitine may provide an effective short term acetyl reservoir and thereby "buffer" the accumulation of acetyl CoA (12). The data in the present study are consistent with this hypothesis. However it is also possible that the formation of acetyl carnitine may represent a physiological control on the rates of fatty acid synthesis and oxidation in heart muscle.

ACKNOWLEDGMENTS

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REFERENCES

1. Friedman, S., and G. Fraenkel, Arch. Biochem. Biophys. 59:40 (1955).
2. Bremer, J., J. Biol. Chem. 237:2228 (1962).
3. Norum, K.R., and J. Bremer, Biochim. Biophys. Acta 78:77 (1963).
4. Fritz, I.B., and K.T.N. Yue, Amer. J. Physiol. 206:531 (1964).
5. Bressler, R., and R.I. Katz, J. Biol. Chem. 240:622 (1965).
6. Fritz, I.B., and S.K. Shultz, Ibid. 240:2188 (1965).
7. Barker, P.J., N.J. Fincham and D.C. Hardwick, Biochem. J. 110:739 (1968).
8. Beenakkers, A.M., and M. Klingenberg, Biochim. Biophys. Acta 84:205 (1964).
9. Lowenstein, J.M., in "Recent Research on Carnitine," Edited by G. Wolf, Massachusetts Institute of Technology Press, 1965, p. 97.
10. Norum, K., Biochim. Biophys. Acta 105:506 (1965).
11. Bremer, J., in "Cellular Compartmentalization and Control of Fatty Acid Metabolism," Edited by F.C. Gran, Academic Press, New York, 1968, p. 65.
12. Pearson, D.J., and P.K. Tubbs, Biochem. J. 105:953 (1967).
13. Rodis, S.L., P. D'Amato, E. Koch and G.V. Vahouny, Proc. Soc. Exp. Biol. Med. 133:1070 (1970).
14. Lowry, O.H., N.J. Rosenbrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193:265 (1951).
15. Abraham, S., K.J. Matthes and I.L. Chaikoff, Ibid. 235:497 (1957).

16. Folch, L., J. Lees and G.H. Sloane-Stanley, Ibid. 226:497 (1957).
17. Baillie, L.A., Inter. J. Appl. Radiat. Isotopes 8:1 (1960).
18. Bremer, J., J. Biol. Chem. 238:2774 (1963).
19. Fisher, R.A., "Statistical Methods for Research Workers," Oliver and Boyd, Edinburgh, 1938.
20. Fritz, I.B., and M.P. Hsu, J. Biol. Chem. 242:865 (1967).
21. Wittels, B., and R. Bressler, J. Lipid Res. 6:313 (1965).
22. Marquis, N.R., R.P. Francescone and C.A. Villee, Adv. Enzyme Reg. 6:31 (1968).
23. Beenakkers, A.M.T., and P.T. Henderson, Eur. J. Biochem. 1:187 (1967).
24. Norum, K.R., and J. Bremer, J. Biol. Chem. 242:407 (1967).
25. Marquis, N.R., and I.B. Fritz, Ibid. 240:2193 (1965).

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Isolation of Brassicasterol from Steam Deodorizer Distillate of Rapeseed Oil: Some Properties of Its Acetate Tetrabromide and Its Reduction to 22,23-Dihydrobrassicasterol[1]

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ABSTRACT

Brassicasterol (5,22-ergostadien-3β-ol) was isolated from the steam deodorizer distillate of rapeseed oil and purified by acetylation, bromination, chromatography on 20% $AgNO_3/SiO_2$ columns and hydrolysis. Brassicasteryl and stigmasteryl (5,22-stigmastadien-3β-ol) acetates were brominated, and the yields of products and solubilities of the tetrabromides from the two steryl acetates were compared. Stigmasteryl acetate tetrabromide is less soluble than the corresponding brassicasteryl derivative; yet the latter precipitates selectively during bromination of a mixture of the two steryl acetates. This is explained on the basis of the stereochemistry of the bromine atoms in the side chains of the two steryl acetate tetrabromides. Hydrogenation of brassicasteryl acetate over Raney nickel gave the 22,23-dihydro derivative in excellent yield. The latter was separated from small amounts of ergostanyl acetate on a 20% $AgNO_3/SiO_2$ column.

INTRODUCTION

Brassicasterol was first obtained by Windaus and Welsch in 1909 (1) from the phytosterol fraction of rapeseed oil, and this source was used in two more recent isolations (2,3). The sterol has also been isolated from molluscs (4) and synthesized from ergosterol by two different procedures (5,6).

The sterols present in very small quantities in crude vegetable oils are concentrated during the steam deodorizing process and appear as substantial fractions of the deodorizer distillates (7-9). Our work with *Drosophila* (10) requires brassicasterol and its 22,23-dihydro derivative, so we chose the deodorizer distillate obtained during the commercial processing of rapeseed oil as a convenient source for these two compounds.

22,23-Dihydrobrassicasterol has been prepared by hydrogenation of the *i*-sterol methyl ether derived from brassicasterol (11,12), from ergosterol by two different multistep syntheses (5,6), from 3β-methoxy-5-cholenoyl chloride and an optically active cadmium reagent (13), and from pregenolone acetate and an optically active Grignard reagent (14).

The availability of pure brassicasterol together with the successful hydrogenation of stigmasterol to its 22,23-dihydro derivative in good yield over Raney nickel (15) prompted us to investigate the preparation of 22,23-dihydrobrassicasterol by direct hydrogenation of the parent sterol.

EXPERIMENTAL PROCEDURES

Methods and Materials

The gas liquid chromatography (GLC), thin layer chromatography (TLC) and titration of unsaturation with bromine of sterols and their esters has been described (16). Brassicasteryl acetate separates readily from a mixture of 22,23-dihydrobrassicasteryl acetate and ergostanyl acetate by GLC on a 5% OV-101 column; the latter two, however, have identical retention times. They were separated on $AgNO_3$/alumina 2:3 thin layer plates (17) with Skellysolve B (petroleum ether, bp 65-67 C) as solvent. Ethyl acetate was distilled before use; triethylamine (MCB) was used as received. Raney nickel (no. 28 active catalyst in water, W.R. Grace and Co., Raney Catalyst Div., Chattanooga, Tenn.) was solvent-exchanged through ethanol and ethyl acetate immediately before use. Melting points were taken in vacuo and are corrected.

Isolation of Sterols

Ten gallons of rapeseed oil steam deodorizer distillate (Fig. 1A) (Canada Packers, Ltd., Montreal) were filtered in two batches at room temperature through four large (3 liter), coarse porosity, sintered glass filters with an oil pump vacuum. Each filtration was hastened by scraping the surface of the funnel twice a day with a large flat-ended spatula. After 15 days (each batch), the precipitates on the four filters (ca. 5 liters total) were washed with an equal volume of ether to remove adhering oil, followed by a wash with absolute ethanol. The original fil-

[1]Contribution No. 2077, Arizona Agricultural Experiment Station.

[2]Presented at the AOCS Meeting, New Orleans, April 1973.

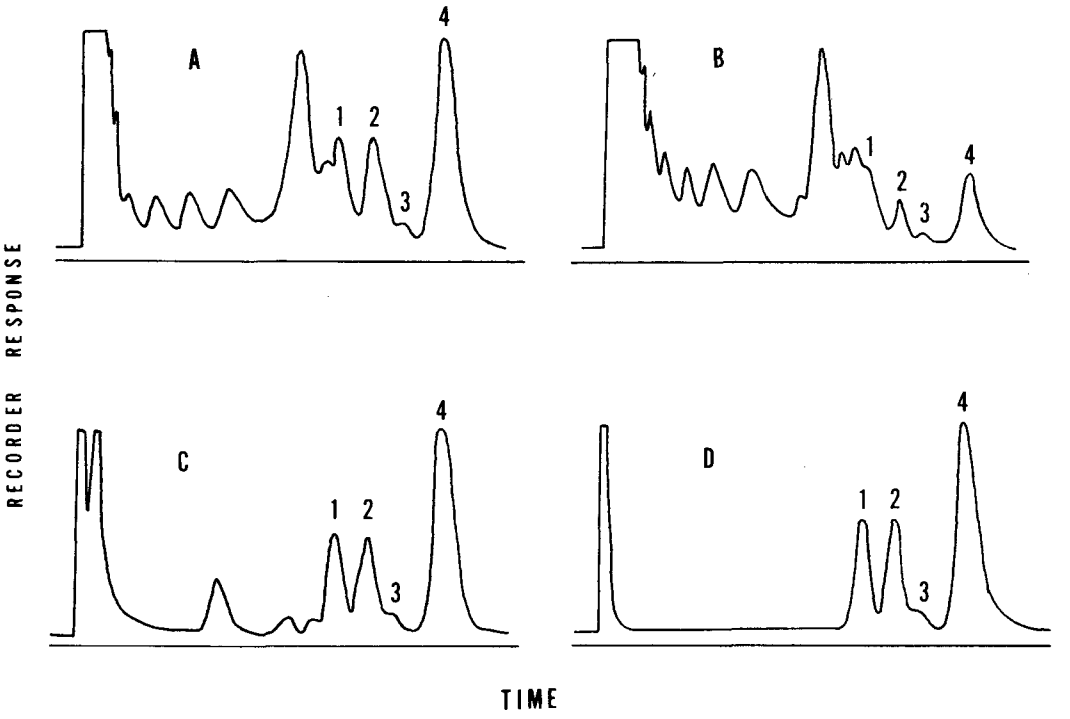

FIG. 1. Gas liquid chromatographic separation diagrams: (1) brassicasterol, (2) campesterol, (3) stigmasterol, (4) sitosterol. A. Crude rapeseed oil steam deodorizer distillate. B. Filtrate from A. C. Filter cake from A. D. Phytosterol mixture obtained from C by crystallization from toluene and isopropanol.

trates and washes (Fig. 1B) were discarded and the precipitates (Fig. 1C) combined in 16 liters hot $CHCl_3$ and filtered to remove a black, gritty material. The $CHCl_3$ was evaporated and the residue crystallized from 4 liters toluene and from isopropanol to yield 1690 g sterol mixture (Fig. 1D).

Acetylation

The sterol mixture was acetylated in six batches on steam baths in the hood with an equal weight of acetic anhydride and sufficient benzene to provide initial homogenity. When the mixtures had evaporated to dryness, the products were washed with methanol in a blender, filtered and dried in air; yield: 1540 g.

Bromination

The steryl acetates (100 g) in 550 ml ether were stirred on an ice bath while 500 ml 10% Br_2 in acetic acid was added dropwise during 30 min. The mixture was then stirred at room temperature for 3 hr and placed in a cold room (4 C) overnight. The precipitate was filtered in the cold, washed with acetic acid and methanol, and air-dried to yield 28 g crude brassicasteryl acetate tetrabromide.

The tetrabromides from 1540 g steryl acetates were combined and stirred with ether (1 liter/250 g), filtered and the procedure repeated. The resulting ether-insoluble precipitate (375 g) was boiled with 2.5 liters acetone, cooled and filtered to yield 344 g purified brassicasteryl acetate tetrabromide.

Debromination

The acetate tetrabromide (100 g) was refluxed with 100 g Zn dust in 1.5 liters 1:1 ethanol-acetic acid for 4 hr. The mixture was decanted from unreacted Zn, the latter washed with a small amount of ether and the combined solutions placed in a cold room overnight. The resulting precipitate was filtered and washed with methanol to give 47.5 g crude brassicasteryl acetate. An additional 4.5 g of a mixture of steryl acetates and free sterols was obtained by addition of water to the filtrate. In all, 170 g brassicasteryl acetate containing ca. 12% stigmasteryl acetate, 8% sitosteryl acetate and 2% campesteryl acetate (Fig. 2A) was isolated from 10 gal rapeseed oil steam deodorizer distillate.

Purification of Brassicasteryl Acetate

A solution of 300 g $AgNO_3$ in 4.5 liters water was mixed with 1500 g silica gel (Mallinckrodt Silicic Acid, A.R., 100 mesh) and 600 g

Celite (Johns-Manville). The mixture was dried on a steam bath for 2 days and in an oven at 110 C overnight. One kilogram was screened (80 mesh), slurried in Skellysolve F-dry diethyl ether 100:1, and poured into a large chromatographic tube (adsorbent dimensions 6 x 85 cm). Ten grams of the crude brassicasteryl acetate mixture (Fig. 2A) was placed on the column in a small volume of benzene and eluted with Skelly F-ether 100:1. After 14 liters had eluted from the column, the steryl acetates began to emerge. Fractions (500 ml) were collected, evaporated and analyzed by GLC. After elution of 2.32 g of sitosteryl, campesteryl and stigmasteryl acetates, fractions rich in brassicasteryl acetate were collected (1.92 g). Stigmasteryl acetate was identified by its melting point (143.5-144.5 C, lit. [18] 144 C) and IR spectrum (superimposable on that of an authentic specimen). When stigmasteryl acetate was no longer evident in the eluate (GLC), the column was eluted with ether to yield 5.61 g brassicasteryl acetate. The 1.92 g of brassicasteryl acetate-rich fraction was rechromatographed on a smaller (250 g) column in the same way to yield an additional 1.53 g of product. The combined materials (7.14 g) were recrystallized from isopropanol to yield 6.25 g brassicasteryl acetate, mp 157.5-158.5 C (Fig. 2B) (highest melting point reported: 158-159 C [6]); double bonds by bromine titration (16): 2.14/mol.

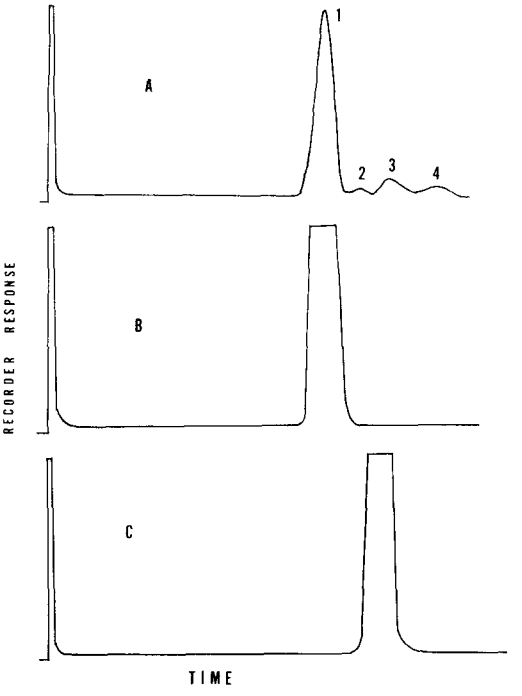

FIG. 2. Gas liquid chromatographic separation diagrams. A. Crude brassicasteryl acetate obtained from ID by bromination and debromination of the insoluble steryl acetate tetrabromides. B. Purified brassicasteryl acetate. C. Purified 22,23-dihydrobrassicasteryl acetate.

Brassicasterol

The acetate (16 g) was hydrolyzed for 2 hr on the steam bath with 900 ml 5% KOH in ethanol. After cooling and one recrystallization from ethanol, 13.4 g brassicasterol was obtained, mp 150-151 C (highest melting point reported: 151 C [6]); double bonds by bromine titration (16): 2.18/mol.

Preparation of Stigmasteryl and Brassicasteryl Acetate Tetrabromides and Determination of their Solubilities

Stigmasteryl acetate (13.5 g, 29.7 mmol) in 180 ml ether was treated with Br_2 (11 g, 69 mmol) in 90 ml acetic acid on an ice bath, and the mixture was kept in the cold room overnight. The precipitate was washed with ether, refluxed with 300 ml ether, filtered and dried to yield 9.15 g (40%) stigmasteryl acetate tetrabromide. The latter was debrominated with 10 g Zn dust in 80 ml acetic acid and the product crystallized from ethanol to yield 4.1 g (78% from tetrabromide, 31% overall) stigmasteryl acetate.

Brassicasteryl acetate (3.1 g, 7 mmol) in 40 ml ether was treated in a like manner with Br_2 (2.4 g, 15 mmol) in 20 ml acetic acid to yield 2.9 g (54%) brassicasteryl acetate tetrabromide.

Samples (ca. 1 g) of the acetate tetrabromides of stigmasterol and brassicasterol were stirred with the bromination reaction solvent (ether-acetic acid 2:1) and with the wash solvent (ether) at room temperature and in the cold room for 18 hr. The stirring was stopped, and a 25 ml aliquot of each run was removed and evaporated in vacuo in a tared flask. The solubilities thus obtained are given in Table I.

Bromination of Mixtures of Brassicasteryl and Stigmasteryl Acetates

Two by weight mixtures of brassicasteryl and stigmasteryl acetates were prepared, 1:1 and 6.7:1, respectively. A sample of each mixture (1.32 g, 3 mmol) was treated with either 1.5 or 2.2 equivalents of Br_2/mol in the usual way, and the sterol acetate tetrabromide mixtures isolated. The yields of precipitates from the various runs and the composition of the products as determined by quantitative GLC analysis after debromination are given in Table II.

Hydrogenation of Brassicasterol

Brassicasterol (0.399 g, 1 mmol) was added

TABLE I

Solubilities of Brassicasteryl and Stigmasteryl Acetate Tetrabromides

Sample[a]	Solvent	Solubilities, g/100 ml solvent 25 C	4 C
Brassicasterol	2:1 Ether-HOAc	0.280	0.238
	Ether	0.387	0.324
Stigmasterol	2:1 Ether-HOAc	0.128	0.084
	Ether	0.218	0.157

[a]Acetate tetrabromides.

to 0.8 ml Raney nickel slurry in 50 ml ethyl acetate and 1 ml triethylamine, and hydrogenated until 1 mmol H_2 had been absorbed (mercury filled gas buret). The product showed no brassicasterol by GLC, a trace of ergostanol by thin layer chromatography (TLC) and contained 0.98 double bonds per mole of sterol by titration with bromine.

Hydrogenation of Brassicasteryl Acetate

Raney nickel (7 ml slurry) was equilibrated with hydrogen in 500 ml ethyl acetate for 1 hr, after which 4.40 g (10 mmol) brassicasteryl acetate was added. Hydrogen uptake ceased after 10.2 mmol had been absorbed (Fig. 3); the reaction mixture was then filtered and evaporated. The residue contained no brassicasteryl acetate (GLC), a small amount of ergostanyl acetate (TLC), and gave 0.929, 0.933 double bonds per mole steryl acetate by bromine titration.

Purification of 22,23-Dihydrobrassicasteryl Acetate

A 20% $AgNO_3$/silica gel column was prepared with Skellysolve F-diethyl ether 100:1 as described above. Crude 22,23-dihydrobrassicasteryl acetate (10 g) was placed on the column in a minimum quantity of benzene and eluted with the 100:1 solvent. After 10 liters had passed through the column, ergostanyl acetate began to emerge. When it was no longer evident in the eluate (TLC), the column was eluted with ether to yield 8.15 g 22,23-dihydrobrassicasteryl acetate, mp 145-146.5 C after recrystallization from ethanol (Fig. 2C).

The products from three such column runs were combined (24 g) and recrystallized from ethanol to yield 21.5 g 22,23-dihydrobrassicasteryl acetate, mp 147.5-148.5 C (Table III), 0.984, 0.991 double bonds per mole by bromine titration.

22,23-Dihydrobrassicasterol

The acetate (13.5 g) was hydrolyzed with 5% KOH in ethanol (1 liter) and the product recrystallized once from ethanol to yield 10.2 g 22,23-dihydrobrassicasterol, mp 159.5-160.5 C (Table III); 1.002, 0.994 double bonds per mole by bromine titration. A benzoate (Table III) was prepared in the usual way.

RESULTS AND DISCUSSION

Brassicasterol occurs together with stigmasterol, campesterol and sitosterol in the steam deodorizer distillate from the commercial processing of rapeseed oil (Fig. 1A). The isolation of the phytosterol fraction (Fig. 1C) was simply accomplished by filtration of the semisolid distillate as obtained. Although a portion of the sterols was lost in the filtrate and washings of the precipitate (Fig. 1B), the ready availability of the inexpensive starting material made extensive work-up procedures unprofitable at this point.

Brassicasterol was readily separated from the bulk of the campesterol and sitosterol by virtue of the insolubility of its acetate tetrabromide

TABLE II

Bromination of Mixtures of Brassicasteryl and Stigmasteryl Acetate

	Brassicasteryl-stigmasteryl ratio (start) 1:1		6.7:1	
Mol Br_2/mol acetate	1.5	2.2	1.5	2.2
Tetrabromide precipitate, % yield	20	57	23	59
Brassicasteryl-Stigmasteryl ratio in precipitate	3.3:1	1.2:1	28:1	10:1
Brassicasteryl-stigmasteryl ratio in product recovered from filtrate	0.6:1	0.5:1	5:1	3:1

(1). Since stigmasterol also forms an insoluble acetate tetrabromide, separation of brassicasterol from this sterol is more difficult. Crystallization of the acetate mixture derived from the large scale brominations-debrominations (Fig. 2A), the free sterols derived from this mixture or the acetate tetrabromides of the sterols from numerous solvents failed to give pure brassicasterol. Some purification was achieved by a repetition of the bromination-debromination procedure (80 → 90% pure brassicasterol), but this was very wasteful; ca. 50% of the starting material was lost in the process (19,20).

Chromatography of 10 g batches of the crude brassicasteryl acetate on 1 kg 20% $AgNO_3$/silica gel (3) with 1% ether in low boiling petroleum ether (Skellysolve F) was finally used as the best way to obtain gram quantities of brassicasterol free from stigmasterol and traces of campesterol and sitosterol. The average yield of 6.25 g pure brassicasteryl acetate from 10 g crude mixture represents an 80% recovery of the sterol from the mixture. Both brassicasterol and its acetate consumed close to 2.15 mol Br_2 per mole of sterol, as did stigmasterol and its esters in a standardized titration procedure (16).

A number of experiments were performed to explain the results obtained from bromination of crude brassicasteryl-stigmasteryl acetate mixtures. We had found in numerous trials (unpublished work) that the tetrabromide acetate precipitates were always enriched in the brassicasterol derivative when compared to the starting material. Determination of the solubilities of the two tetrabromides (Table I) showed that this property does not provide the answer; the stigmasterol derivative had the lesser solubility under all conditions.

Bromination of stigmasteryl and brassicasteryl acetate individually and in two synthetic mixtures suggested reasons for the above observations. The yield of solvent-insoluble acetate tetrabromide was 40% from stigmasterol and 54% from brassicasterol. In addition, when

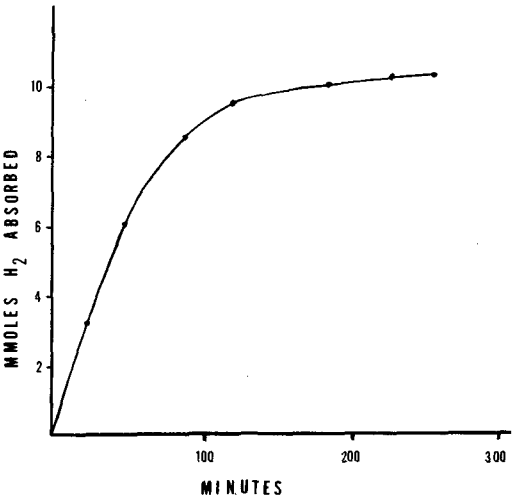

FIG. 3. Hydrogenation of 10 mmol brassicasteryl acetate in ethyl acetate over Raney nickel.

mixtures of the two acetates were brominated with only 1.5 mol Br_2 per mole steryl acetate, brassicasteryl acetate tetrabromide was largely enriched in the resulting precipitates (Table II); whereas when an excess of Br_2 was added (2.2 mol Br_2 per mole acetate), the enrichment was much smaller (Table II).

Theoretically, four isomers of each sterol acetate tetrabromide are possible by *trans* addition (21) of bromine to the Δ^5 and Δ^{22} double bonds: 5α, 6β, 22R, 23S; 5α, 6β, 22S, 23R; 5β, 6α, 22R, 23S; and 5β, 6α, 22S, 23R. However the preferential attack of Br_2 on the Δ^5 bond in a $\Delta^{5,22}$ diene (22) from the α-side of the sterol molecule (23), together with the low temperatures maintained in this work during bromination and work-up to inhibit 5α,6β → 5β,6α isomerization (24), suggests that the 3β-acetoxy-5α,6β-dibromo configuration is the same in both brassicasteryl and stigmasteryl acetate tetrabromides and that each sterol yields only two isomers. The results are then best explained on the basis of the stereochem-

TABLE III

Melting Points (C) of 22,23-Dihydrobrassicasterol, Its Acetate and Benzoate

Source	Sterol	Acetate	Benzoate
Fernholz and Ruigh (11)	158	145	162
Barton and Robinson (6)	146-148	152-154	
Clayton and Bloch (12)	155-156		
Thompson et al. (5)	158-159	146-148	
Martinez et al. (13)	148-149	153-155	
Ikan et al. (14)		140	
Present work[a]	159.5-160.5	147.5-148.5	164-165

[a]In vacuo, corrected.

istry of the side chain in the two sterols.

The rates of formation and the solubilities of the four tetrabromo derivatives are probably quite different. From the data in Table II, it is reasonable to conclude that the rate of bromination of the Δ^{22} bond in brassicasteryl acetate is greater than that of the stigmasteryl derivative or the formation of an insoluble 5α, 6β, 22ξ, 23ξ tetrabromide in the former case is sterically favored. The enrichment of brassicasterol in precipitates obtained by bromination of mixtures of the two steryl acetates is then a function of the rates of reaction of the Δ^{22} bond with bromine in the two compounds, together with the relative insolubility of a sterically favored brassicasteryl acetate tetrabromide.

Hydrogenation of brassicasterol or its acetate over Raney nickel is a convenient way to prepare 22,23-dihydrobrassicasterol. The sterol and its acetate contained 1.00 double bond per mole by a standardized bromine titration; in this way it resembles cholesterol and sitosterol (16). The melting points observed in the present work are in accord with some of those recorded in the literature, but at variance with others (Table III). It is possible that the compounds prepared in three earlier studies (6,13,14) either were not pure or were isomers of the desired dihydrobrassicasterol.

The hydrogenations of brassicasteryl acetate (Fig. 3) were much more facile than those observed for stigmasterol or its esters (15). In the latter cases, 20-30% of stigmastanol formed when the reductions were run until almost all stigmasterol had been consumed. By contrast, only 3-7% of ergostanyl acetate was formed in reaction mixtures that contained not a trace of brassicasteryl acetate. This increased activity of the Δ^{22} bond in brassicasteryl acetate over that in the stigmasteryl derivative was also observed during bromination of the two steryl acetates.

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REFERENCES

1. Windaus, A., and A. Welsch, Ber. 42:612 (1909).
2. Fernholz, E., and H.E. Stavely, J. Amer. Chem. Soc. 61:142 (1939).
3. Thompson, M.J., S.R. Dutky, G.W. Patterson and E.L. Gooden, Phytochem. 11:1781 (1972).
4. Bergman, W., and R.C. Ottke, J. Org. Chem. 14:1085 (1949).
5. Thompson, M.J., C.F. Cohen and S.M. Lancaster, Steroids 5:745 (1965).
6. Barton, D.H.R., and C.H. Robinson, J. Chem. Soc. 1954:3045.
7. Hoffman, R.L., C.D. Evans and J.C. Cowan, JAOCS 41:116 (1964).
8. Nelson, J.P., and A.J. Milun, Ibid. 45:848 (1968).
9. Kircher, H.W., and F.U. Rosenstein, Lipids 8:97 (1973).
10. Kircher, H.W., and W.B. Heed, Adv. Phytochem. III:191 (1970).
11. Fernholz, E., and W.L. Ruigh, J. Amer. Chem. Soc. 62:3346 (1940).
12. Clayton, R.B., and K. Bloch, J. Biol. Chem. 238:586 (1963).
13. Martinez, A., A. Romeo and V. Tortorella, Gazz. Chim. Ital. 97:96 (1967).
14. Ikan, R., A. Markus and E.D. Bergmann, Steroids 16:517 (1970).
15. Kircher, H.W., and F.U. Rosenstein, Lipids 8:101 (1973).
16. Rosenstein, F.U., and H.W. Kircher, Ibid. 8:107 (1973).
17. Allais, J.P., and M. Barbier, Qual. Plant. Mater. Veg. 16:215 (1968).
18. Shoppee, C.W., "Chemistry of the Steroids," Second edition, Butterworths, London, 1964, p. 63.
19. Campbell, J.A., D.A. Shepherd, B.A. Johnson and A.C. Ott, J. Amer. Chem. Soc. 79:1127 (1957).
20. Donia, R.A., A.C. Ott and N. Drake, Anal. Chem. 29:464 (1957).
21. Wheland, G.W., "Advanced Organic Chemistry," Second edition, Wiley and Sons, New York, 1949, p. 290.
22. Fernholz, E., Ann. 507:128 (1933).
23. Barton, D.H.R., and E. Miller, J. Amer. Chem. Soc. 72:1066 (1950).
24. Barton, D.H.R., and A.J. Head, J. Chem. Soc. 1956:932.

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Use of a Bile Duct T-Cannula as a New Technique for Studying Bile Acid Turnover in the Rat

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ABSTRACT

A new technique using T-cannulated bile ducts has been developed for studying bile acid turnover in the rat by isotope dilution. Applicability of this technique to study the effect of diet on cholate and chenodeoxycholate metabolism is suggested. In tests with the method, the rate of cholate synthesis for rats fed colony diet was twice that for rats fed semisynthetic diet. When sodium glycocholate was fed in the latter diet the half-life of cholate decreased; the secretion rate of deoxycholate and cholate increased without affecting chenodeoxycholate. Although curves for cholate log specific activity vs. time were linear, those obtained simultaneously for chenodeoxycholate were nonlinear. A double exponential curve was obtained for chenodeoxycholate of colony-fed rats. Separation of free deoxycholate and chenodeoxycholate was improved through continuous development thin layer chromatography.

INTRODUCTION

The only method available for studying bile acid turnover in the rat has been to analyze fecal excreta for label after administering a labeled bile acid (1). With this method fecal radioactivity also includes metabolites of the bile acid studied; hence turnover constants obtained in this manner are really excretion constants. Another disadvantage is that pool size cannot be obtained from the kinetic data. The drop in specific activity of a bile acid in bile has been studied in man (2,3), and true turnover constants and pool sizes are obtained. For the rat no similar techniques have been refined for repetitive collection of bile without disturbing the normal enterohepatic circulation of the bile acids or their pool size. This report describes such a technique in combination with continuous development thin layer chromatography (TLC) as a direct means to obtain data on turnover of individual bile acids.

MATERIALS AND METHODS

T-Cannula Construction

Silastic medical-grade tubing (Dow Corning Corp., Midland, Mich.) was used to make the T-cannula. Tubing A (0.040 in. ID, 0.085 in. OD) was cut to 7.5 cm lengths, and tubing B (0.020 in. ID, 0.037 in. OD) to 15 cm lengths. Approximately 1 cm from an end of tubing A, two holes were notched with a scalpel on opposite sides no larger than needed to accommodate tubing B without pinching it off (Fig. 1). A small notch was cut 6 cm from one end of tubing B, and both the shorter end (B_1) and the longer end (B_2) were bevelled at a 45° angle. Tubing B was then threaded through the holes of tubing A, and the notch in tubing B was adjusted to face the lumen of the longer end of tubing A. The short end of tubing A was cut to ca. 0.3 cm and filled with epoxy resin, which also was applied to the outside of the T junction. The resin was allowed to set for at least 24 hr, and the joint was tested for leaks or

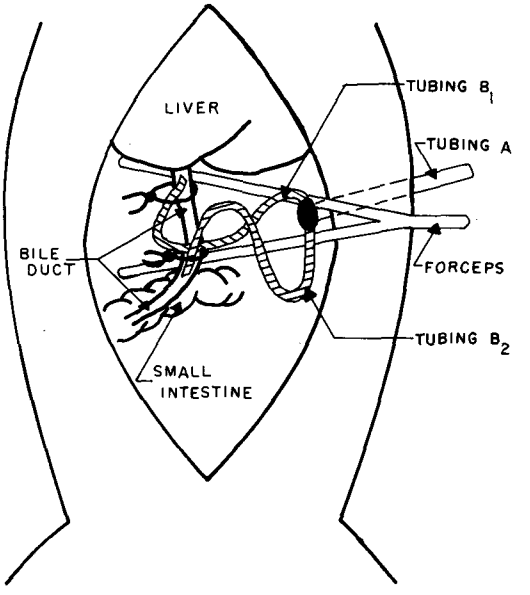

FIG. 1. T-Cannula insertion into bile duct of rat. Details of operation described in text.

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obstructions by injecting water through it.

Surgical Procedure

Rats weighing 300 g or more were fasted overnight, anesthetized with Nembutal (3.5 mg/100 g body wt), and hair was clipped from the middle trunk. A median incision ca. 6 cm long was made in the abdomen just below the xiphisternum. With a scalpel a small slit was made in the rat's left side below the ribs. The open end of tubing A of the T-cannula was then exited through the hole for half its length. The bile duct was exposed close to the liver, stretched with forceps and cleared of fat particles. With surgical thread in position a small incision was made in the upper part of duct; tubing B_1 was inserted upward and tied into place (Fig. 1). The lower incision was then made; tubing B_2 was inserted, pushed downward and tied into place. Tubing A was then pulled from outside the body until the junction of the T-cannula was against the inner body wall. From the outside tubing A was sewed into place using a purse string stitch through skin and muscle layers. Protruding tubing A was trimmed to ca. 4 cm and a Teflon-coated, brass rod (16 gauge) was used to stop bile flow. Tubing B_2 was visually checked for bile flow. Then 3 drops of 0.1% tetracycline aqueous solution were placed into the abdominal cavity, and the incised wound was sutured. Collodion (Mallinckrodt Chemical Works, St. Louis, Mo.) was used to coat both wounds. After the collodion dried, a 2 in. wide bandage was applied to prevent damage to the cannula. To prevent chewing of cannula and sutures upon partial restoration of usual activity, a rubber collar was placed around the rat's neck 24 hr after surgery. A disc 7.5 cm in diameter was cut from 3 mm firm rubber gasket stock, and a center hole 2.5 cm or more in diameter was made with a cork borer. A radial slit permitted opening of the collar for placement. The rat wearing the collar was allowed to run freely in a plastic case with wire screen bottom.

Bile Collection

Bile flow rates from the T-cannula were similar to those from a continuously flowing cannula. For a 300 g rat the rate was ca. 0.3 ml/15 min; it was slightly higher when bile salts were fed. After a 3 or 4 day recovery period, animals that demonstrated normal flow and had no apparent abnormalities (jaundice, infection, etc.) were injected by cardiac puncture with a tracer amount of [carboxyl-^{14}C]chenodeoxycholic acid (0.33 μCi) and [G-^{3}H]cholic acid (1.54 μCi) in 0.25 ml 40% ethanolic saline. Both [carboxyl-^{14}C]chenodeoxycholic acid (35.8 mCi/mM, International Chemical and Nuclear Corp., Irvine, Calif.) and [G-^{3}H]cholic acid (2.12 Ci/mM, New England Nuclear, Boston, Mass.) were 92+% radiochemically pure by thin layer chromatography (see bile analysis). Since the impurities gave no discrete band but were diffuse, the compounds were used as purchased. In most cases 15 min samples were collected 24 hr after injection from ether-anesthetized rats after discarding the first 2 drops of bile. All samples were then taken daily at the same hour for a total of 5 days. They were frozen immediately and held for analysis. The rats were checked 2 days later for normal bile flow and physical condition to confirm that samples taken on the fifth experimental day were acceptable. Any samples taken from an animal that later appeared abnormal were discarded.

Bile Analysis

After thawing, the bile samples (ca. 0.3 ml) were extracted with 5 ml ethanol (10 min at 60 C); the proteinaceous precipitate was reextracted with 5 ml acetone-ethanol 1:1, and the combined extracts were then taken to dryness in a polypropylene centrifuge cup in a 60 C oven. To the dry residue 3 ml 2 N aqueous NaOH was added, and the mixture was heated 3 hr at 15 lb steam pressure to saponify. After cooling, 0.6 ml concentrated HCl was added to give a pH of ca. 1.0. The solution was transferred to a separatory funnel with the aid of 2 ml distilled H_2O and extracted three times with 5 ml portions of ether. The combined extracts were taken to dryness and the free bile acids quantitatively transferred with methanol to thin layer plates (0.5 mm, Silica Gel G) that were activated at 80 C. To obtain continuous development, a sheet of filter paper (Whatman 3-M, 14 x 23 cm) was attached to the leading edge of the plate by clamping a glass rod to the plate (Fig. 2). The plates were then placed into developing tanks (two plates per tank) containing isooctane-ethyl acetate-acetic acid 100:50:14. The tanks resided in a chamber, through which air moved at a regulated rate for 6.5 hr at 25 C, and were covered with I-shaped lids to allow the air flow to evaporate solvent from the exposed edge of the filter paper.

On removal from the tanks, the plates were air-dried for 1 hr and sprayed with water in a horizontal position to visualize the bile acid bands. These bands were identified with pure bile acids, a mixture of which (cholate [C], chenodeoxycholate [CDC] and deoxycholate [DC]) was spotted at the origin on one edge of each plate before development. Neutral sterols were not retained on the plate. Bands were outlined while still moist, and the marked

TABLE I

Recovery of Labeled Bile Acids[a,b]

Bile acid	Label recovered, %		Label in DC band, %
	Developed plate[c]	Entire procedure[d]	
C	80.9 ± 0.7	76.6 ± 1.0	---
CDC	90.0 ± 2.1	92.2 ± 2.9	3.2 ± 2.0

[a]Techniques used are outlined in text and values are expressed as mean ± standard deviation.

[b]C, cholic acid; CDC, chenodeoxycholic acid; TLC, thin layer chromatography.

[c]Labeled bile acids, 12,000 dpm, were diluted with 3 mg C or 0.5 mg CDC and applied to TLC plate.

[d]Labeled bile acids, conjugated C or free CDC, were added to 15 min bile specimens.

plates were allowed to air-dry overnight. Each band was scraped into a 25 ml volumetric flask; methanol was added to volume, and samples were allowed to extract overnight. The extract was filtered and 15 ml taken to dryness in a scintillation vial; after adding 1 ml methanol to redissolve the bile acids, toluene scintillation fluid (4.0 g/liter 2,5-diphenyloxazole, 0.1 g/liter 1,4-bis-2-[5-phenyloxazolyl]-benzene) was added for counting (Packard, Tri-carb). Counting efficiencies were determined by the channels-ratio method and dpm were calculated. The remaining filtrate was used for photometric analysis similar to previously established methods (4). Two milliliters CDC or 3 ml DC filtrate was taken to dryness and the residue treated with 4 ml 65% H_2SO_4. Solutions were heated at 60 C for 1 hr and, after cooling with tap water, absorbance was determined for CDC at 380 nm and DC at 385 nm. Both colors were stable for more than 1 hr. For C 0.5 ml filtrate was taken to dryness, the residue treated with 4 ml 65% H_2SO_4, and the mixture allowed to stand at room temperature for exactly 1 hr. Absorbance was determined at 320 nm. From the results of photometric and scintillation analysis, specific activity (dpm/mg) was then calculated.

Diets

Male rats of Wistar strain were fed either the colony diet or a semisynthetic diet. The colony diet (4% fat, 60% carbohydrate, 23% protein and 13% noncaloric) consisted of ground corn, wheat middlings, meat meal and other natural ingredients. The semisynthetic diet consisted (in per cent) of lard (Stark and Wetzel), 5; dextrose, 72; casein, 16; Celluflour, 2; mineral mixture no. 185 (5), 4; and a vitamin mixture that was prepared as a premix with a portion of the casein (6). To this diet 0.5% sodium glycocholate (Nutritional Biochemical Corp., Cleveland, Ohio) was added in some experiments.

RESULTS AND DISCUSSION

Techniques

The type of tubing used to make the T-cannula was very important. The tubing must be flexible for proper insertion and bile flow. While other tubing was tried, only the Silastic tubing met these requirements. The entire surgical operation consumed ca. 15 min. In developing this technique we found that unsuccessful operations (ca. 40%) became evident during the first 3 days; after that more than 80% of the animals appeared normal throughout the period required for an experiment. The collars and cages used prevented both cannula destruction and coprophagy.

Analysis of bile acids for taurocholic acid was reproducible, although recovery was not quantitative (Table I). A similar recovery was obtained with free CDC; samples of the taurine conjugate were lacking. With both C and CDC the major source of loss was extraction from the Silica Gel G. A number of different solvent systems were tried, but none gave better results. While extraction of bile acids with boiling methanol allowed rapid recovery, overnight extraction at room temperature gave comparable results and was more convenient with a large number of samples. Air drying the water-sprayed plates overnight gave better recoveries than heating the plates for 1 hr at 100 C. Poor extraction of bile acids from Silica Gel G has been noted (7).

By continuous development TLC, DC and CDC were separated clearly (Table I and Fig. 2) when applied in amounts in excess of that usually present in 15 min bile samples (Table II). Regulation of conditions was crucial, as increases in temperature, humidity, air flow rate through the chamber and amounts of acetic acid increased the migration rate of all the bile acids. If the rate was too fast or too slow, separation was not as good. We obtained best separation when these conditions were

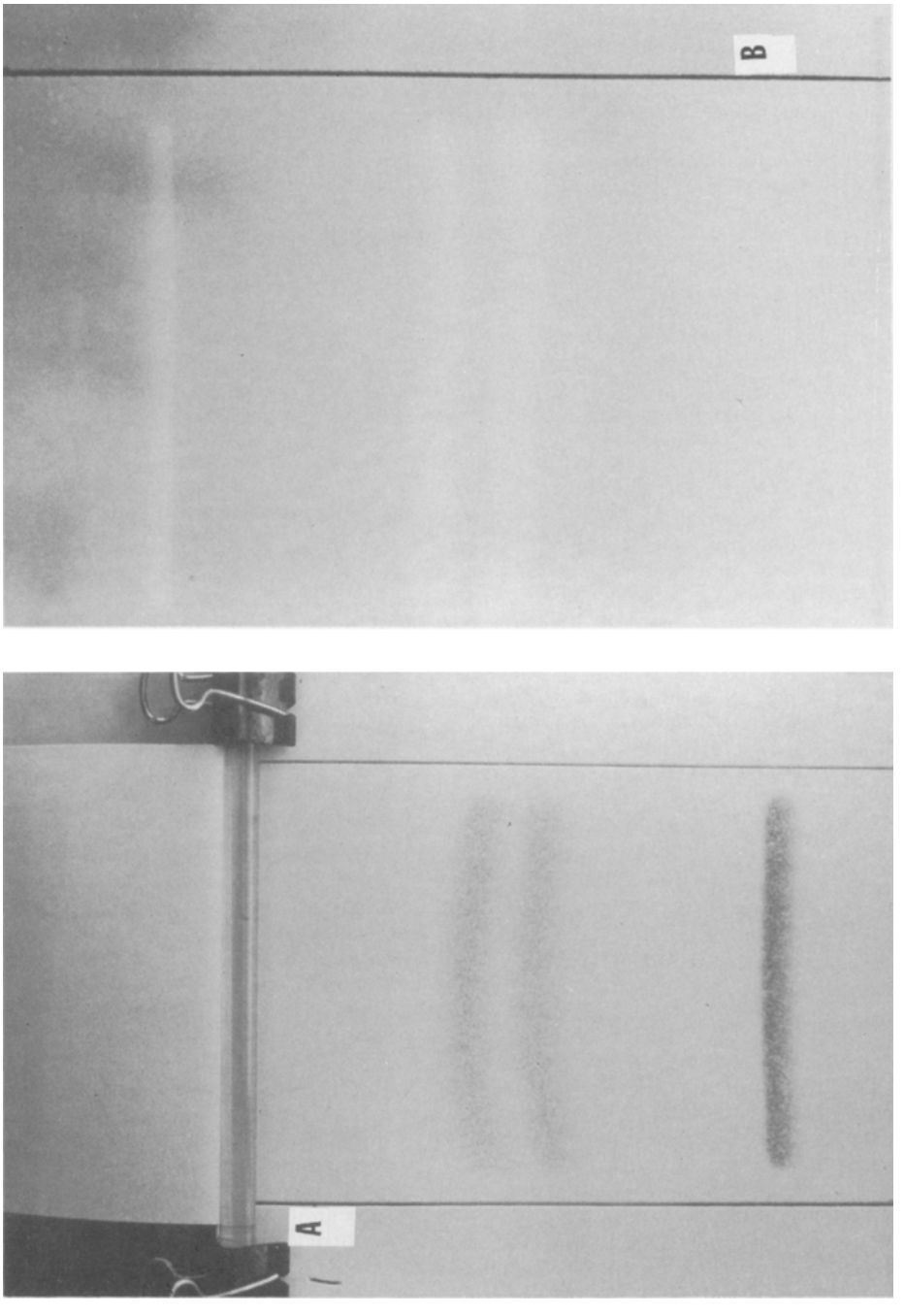

FIG. 2. Relative migration of deoxycholate (DC), chenodeoxycholate (CDC) and cholate (C) when subjected to continuous development thin layer chromatography (TLC). DC, CDC and C (500, 500 and 1000 μg) were applied to 0.5 mm Silica Gel G plates, which were subjected to continuous development TLC using isooctane-ethyl acetate-acetic acid 100:50:14. Developed plates were sprayed with 2% H_2SO_4 in methanol (A) or with water (B) to detect bands. Mobilities relative to DC were 0.82 (CDC) and 0.17 (C).

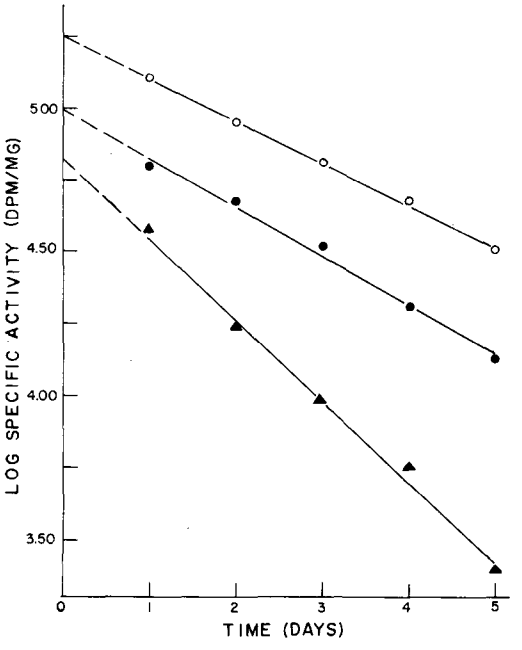

FIG. 3. Decline of cholate specific activity with time as affected by diet. Semisynthetic (○), semisynthetic + bile salts (▲), or colony (●) diets were fed during and 4 weeks previous to experiments in which rats with T-cannulated bile ducts were injected with [G-^{3}H] cholic acid and ^{14}C-labeled chenodeoxycholate simultaneously as a 40% ethanolic saline solution by cardiac puncture. First bile specimen was taken 1 day later, and 15 min bile specimens were then taken daily. Analysis gave SA, which were averaged for each diet and plotted. Number of rats used is shown in Table III.

regulated so that the leading band (DC) migrated ca. 80% up the plate in 6.5 hr. Separations were then substantially improved over those described previously for DC and CDC (8,9).

The cholic acid band was a mixture of trihydroxy bile acids. An extract of this band from rat bile rechromatographed with chloroform-methanol-acetic acid 80:12:3 for a single development (10) showed that β-muricholate was also present. However conditions used for spectrophotometric analysis are very specific for C (4) and, since no interconversion of label between C and β-muricholate was likely (11), the band was used for C analysis without further purification. Gas liquid chromatographic analysis of the eluate from the CDC band showed trace contaminations with DC, which is consistent with the 3% overlap observed by radioisotopic measurement. In our TLC system ursodeoxycholic acid and hyodeoxycholic acid standards separated as bands between C and CDC. It was therefore deduced that the CDC band was free from other common bile acids.

TABLE II

Composition of Rat Bile as Affected by Diet[a,b]

Diet	Mass, μg/15 min			Mass ratio
	DC	CDC	C	C/CDC
Semisynthetic	140	134	1940	16.3
Semisynthetic + bile salts, 0.5%[c]	1080	144	6160	48.9
Colony	319	360	3020	9.6

[a]Rats with T-cannulated bile ducts were fed various diets and 15 min bile specimens collected daily. Masses were determined spectrophotometrically after separation of individual bile acids by continuous development TLC. Values are expressed as averages of individual bile specimens collected throughout experiment.

[b]For abbreviations see Table I.

[c]Commercial sodium glycocholate.

Application of Method

The composition of bile varied with diet fed (Table II). The semisynthetic diet caused a lower total bile acid secretion rate than did our colony diet. Adding commercial bile salts, which contained C, DC and CDC (14:4:1), to the same diet increased both DC and C substantially without affecting CDC.

Kinetic considerations of C metabolism of these rats showed a linear relationship of log SA and time after injecting [G-^{3}H] cholic acid (Fig. 3). This relationship implies first order kinetics

TABLE III

Effect of Diet on Cholate Metabolism[a]

Diet	No. of rats	Half-life, days	Pool size, mg	Synthesis rate, mg/day
Semisynthetic	9	2.1	19.3	6.6
Semisynthetic + bile salts, 0.5%	4	1.1	50.1	---
Colony	10	1.8	34.4	13.4

[a]Values were calculated from plot, log SA of cholate vs. time (Fig. 3) by using least squares equations to obtain slope and ordinate intercept.

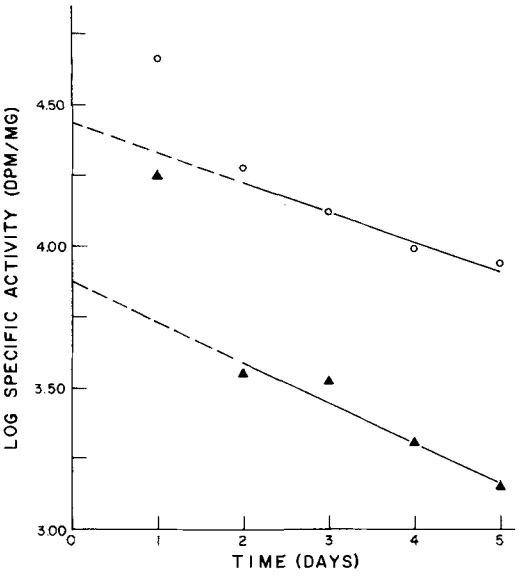

FIG. 4. Decline of chenodeoxycholate (CDC) specific activity with time as affected by diets. By using ^{14}C-labeled CDC in experiments outlined in Figure 3, specific activities of CDC were averaged and plotted by semisynthetic (○) and semisynthetic + bile salts (▲) diets.

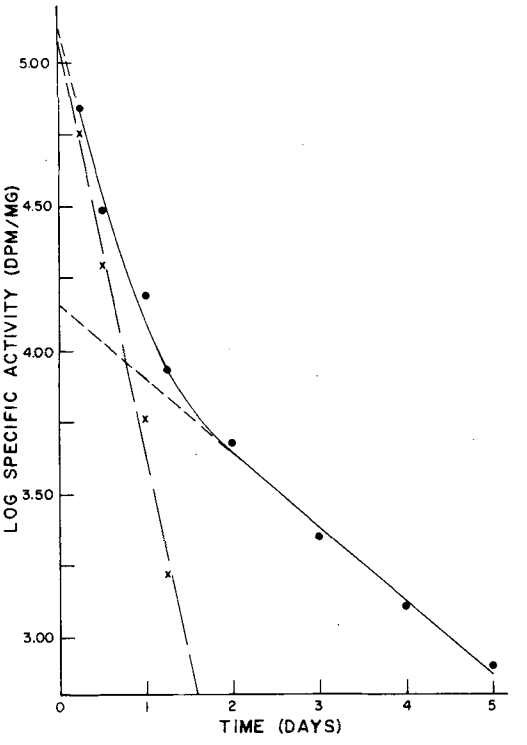

FIG. 5. Decline of chenodeoxycholate specific activity with time for colony diet. Experiment was performed as outlined in Figure 4, except that earlier initial 15 min collections of bile were obtained in separate experiments. Experimental values were determined and plotted (●). Differences of experimental specific activity and antilog of points from extrapolated linear curve gave virtual specific activities, which were plotted (X).

and permits calculation of pool size and half-life of C (Table III). Using a semisynthetic diet appeared to lower C pool size but produced only small changes in half-life. Feeding bile salts expanded the C pool size and increased its turnover (decreased half-life) as expected. The C synthesis rate for colony-fed rats was twice that found in the semisynthetic diet group. Since high exogenous C would distort values for C synthesis, the synthesis rate could not be calculated for the bile salt-supplemented diet. The pool size, half-life and synthesis rate for colony diet were similar to values obtained with a Purina Chow diet (12). Their semisynthetic diets gave a longer half-life than ours unless Celluflour (20%) was added. We added Celluflour (2%) to our diet.

Perhaps more important, the turnover constants for bile acids previously reported for rats have really been excretion constants since they were all based on techniques developed by Lindstedt and Norman for fecal excretion of total radioactivity (1). In contrast to the linear plots obtained with the Lindstedt and Norman Technique (1), our kinetic studies of CDC metabolism showed a linear plot only after the second day (Figs. 4 and 5). This may be of importance in studying CDC metabolism, since our trihydroxy band contained ca. 15 times as much radioactivity as the CDC band when ^{14}C-chenodeoxycholic acid had been injected. One must recognize that excretion constants are concerned with turnover of the entire pool of primary bile acids and their metabolic products, while turnover constants deal with specific bile acids.

For all diets the specific activity of CDC on the 1st day was substantially higher than first order kinetics would indicate. It seemed unlikely that the 1st day value was an artifact of our T-cannula technique, since the kinetics of C (Fig. 3) were determined simultaneously with the same animals. Besides, the pool size, which was calculated from the intercept of the straight line of the last 4 days, was relatively large. Experiments with direct bile duct drainage showed that radioactive CDC, which was injected in the same manner as in the T-cannula experiments, was cleared from the circulatory system rapidly; 95% of the injected radioactivity was cleared in 2 hr and 91% of this amount in the first 30 min. Thus we concluded that the loss of linearity was not attributable to our experimental techniques.

To obtain early responses eight rats from the colony were injected with labeled CDC at the same time of day and bile samples were taken at 6 and 30 hr from three rats, 12 hr from two rats, and 24 and 48 hr from the three others. Using the mean specific activity values in combination with those from the initial experiment with colony fed rats, a nonlinear curve was produced (Fig. 5). This curve was represented by the double exponential equation: $SA_t = 1.24 \times 10^5 e^{-3.33t} + 0.14 \times 10^5 e^{-0.593t}$, where SA_t is specific activity in dpm/mg at any time, t, in days. This equation suggests a two pool model. Calculating the CDC pool size of the injected pool from SA_o gave a value of 5.25 mg.

Although these data represent the first evidence for the existence of a nonlinear plot for CDC or any bile acid, we feel that the Lindstedt and Norman technique lacks the sensitivity to detect nonlinearity for the rat. When the radioactivity of CDC and its trihydroxy metabolites were combined and divided by the CDC mass for each sample, log SA vs. t plot yielded a linear relationship. Thus, when a major liver metabolite of CDC was included, it masked the double exponential curve. It is possible that metabolites also masked this nonlinear curve in determining excretion constants.

We also recognize that the isotope dilution technique has been used in man with no evidence of a nonlinear relationship, but the rat is a unique animal in that it has no gall bladder. The bile acids are continually recycling through the enterohepatic system, with the majority being present in the small intestine at all times. There are also some present in the colon and it has been suggested that two pools may exist in the rat, one in the small intestine, the other in the large intestine (13). While our data do not prove this theory, they suggest that further work in this area might be fruitful. Our techniques for T-cannulated bile ducts and separation of DC and CDC by continuous development TLC should aid in such studies.

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REFERENCES

1. Lindstedt, S., and A. Norman, Acta Physiol. Scand. 38:121 (1957).
2. Lindstedt, S., Ibid. 40:1 (1957).
3. Danielsson, H., P. Eneroth, K. Hellstrom, S. Lindstedt and J. Sjövall, J. Biol. Chem. 238:2299 (1963).
4. Sjövall, J., Clin. Chim. Acta 4:652 (1959).
5. McCollum, E.V., and N. Simmonds, J. Biol. Chem. 33:55 (1918).
6. Quackenbush, F.W., and M.D. Pawlowski, J. Nutr. 72:196 (1960).
7. Kallner, A., Acta Chem. Scand. 21:87 (1967).
8. Wollenweber, J., B.A. Kottke and C.A. Owen, Jr., J. Chromatogr. 24:99 (1966).
9. Eneroth, P.J., J. Lipid Res. 4:11 (1963).
10. Siegfried, C.M., and W.H. Elliot, Ibid. 9:394 (1968).
11. Danielsson, H., and T.T. Tchen, in "Metabolic Pathways," Vol. 2, Edited by D.M. Greenburg, Academic Press, New York, 1968, p. 141.
12. Portman, O.W., and P. Murphy, Arch. Biochem. Biophys. 76:367 (1958).
13. Weiner, I.M., and L. Lack, in "Handbook of Physiology," Vol. 3, American Physiological Society, The Williams and Wilkins Co., Baltimore, Md. 1968, p. 1439.

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Turnover of Bile Acids in the Hypercholesterolemic Rat as Influenced by Saturation of Dietary Fat

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ABSTRACT

Injections of [24-^{14}C]chenodeoxycholate and ^{3}H-cholate were made by heart puncture into 300 g male rats that bore T-cannulas in their bile ducts. The animals had been raised on diet A, containing glucose, cholesterol and cholate, or diet B, containing sucrose and cholesterol; each of the diets contained 5% safflower oil or 5% beef tallow as variables. From analysis of bile samples collected from the T at intervals over a 5 day period, it was observed that the safflower oil group fed diet B had a 17% shorter cholate half-life, a 29% larger cholate pool size and 52% higher rate of cholate synthesis than those fed beef tallow in the same diet. The safflower group fed diet A also had a larger cholate pool size, but synthesis and half-life were obscured by cholate feeding. Chenodeoxycholate turnover data were not obtainable because the decay curves were bimodal for all treatments and hence did not conform to a simple pool model. It is concluded that dietary safflower oil causes more rapid formation of cholate than does dietary beef tallow in the cholesterol-fed rat.

INTRODUCTION

During the last two decades many studies have been performed to elucidate the mechanism by which dietary polyunsaturated fat lowers plasma cholesterol levels. In experiments with humans there is evidence that dietary corn oil produces a shorter cholic acid half-life than dietary butter fat (1) or coconut oil (2). However from studies with rabbits it was concluded that turnover of bile acids is the same whether hydrogenated coconut oil or corn oil is fed (3). In experiments with rats excretory bile acids have been analyzed for mass or recovery of label (4-6), but since direct turnover studies were not performed evidence is lacking concerning the dynamic aspect of bile acid formation in response to polyunsaturate feeding.

Recent success with the use of a T-cannula in this laboratory has made possible the periodic sampling of bile from the rat without disturbing enterohepatic circulation (7). Using this technique on cholesterol-fed rats that had a wide differential in plasma cholesterol levels as a result of dietary differences in polyunsaturates, we have now observed turnover responses following the injection of labeled bile acids. The data show that long term polyunsaturate feeding influences cholate formation.

EXPERIMENTAL PROCEDURES

Weanling male rats of the Wistar strain were grown on two different semisynthetic diets. Diet A was composed of (in per cent): glucose (Cerelose) 72; casein, 15.3; Celluflour, 1.7; vitamin mixture, 1.7; mineral mixture, 3.4; cholesterol, 1.0; sodium glycocholate, 0.4; and either safflower oil or beef tallow, 5.0. The mineral mixture and vitamin mixture including vitamin D (1920 IU/kg) and vitamin E (125 mg/kg) were prepared as described previously (8). Diet B had the same composition, except that it contained sucrose instead of glucose, no sodium glycocholate, and the cholesterol was heated in the fat at 170 C for 1 hr before addition to the diet.

After the animals reached 300 g body wt, they were anesthetized with Nembutal and the T-cannula was inserted in their bile duct (7). After a 2 or 3 day recovery period the diet A animals received simultaneously by cardiac injection 0.5 μCi of [24-^{14}C]-chenodeoxycholic acid (International Chemical & Nuclear) and 1.7 μCi of ^{3}H-cholic acid (New England Nuclear). The diet B rats were divided into two groups; one group received 1.0 μCi of [24-^{14}C]chenodeoxycholic acid and the other 1.5 μCi of ^{3}H-cholic acid.

Bile was collected as 15 min samples by the procedure that has been described, at the following intervals: all of the diet A rats and the diet B rats that were injected with cholate were sampled at 24, 48, 72, 96 and 120 hr after injection. Diet B rats that were injected with chenodeoxycholate were sampled in three series: 6, 30, 54, 78, 102 and 126 hr after injection; 12, 36, 60, 84, 108 and 132 hr after injection; and 24, 48, 72, 96 and 120 hr after injection. Analysis of the bile was performed as described previously (7), except that deoxy-

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TABLE I

Summary of Treatments and Isotope Turnover as Determined from 15 Minute Collections of Bile via T-Cannula[a]

Parameter	Dietary fat, 5%			
	SFO	BT	SFO	BT
	Dietary sugar, 72%			
	Glucose	Glucose	Sucrose	Sucrose
	Dietary cholate, %			
	0.4	0.4	None	None
Cholate in bile, mg/15 min	2.67	2.33	1.85	1.60
CDC in bile, mg/15 min	0.48	0.39	0.71	1.02
Ratio, C/CDC	5.6	5.9	2.6	1.6
^{3}H-Cholate injected, dpm x 10^{-6}	3.8	3.8	3.2	3.2
[4-^{14}C]CDC injected, dpm x 10^{-6}	1.11	1.11	2.22	2.22
Cholate in bile, SR, dpm x 10^{-5}	0.71	0.96	1.95	2.51
Cholate pool size, mg	53.8	40.0	16.4	12.7
Cholate decay slope, K, days^{-1}	0.98	1.06	0.89	0.76
Cholate synthesis rate, mg/rat/day	—	—	14.6	9.6
Cholate half-life, $T_{1/2}$, days	0.70	0.65	0.78	0.91

[a]SFO, safflower oil; BT, beef tallow; CDC, chenodeoxycholic acid; SR, specific radioactivity. Data are calculated from mean values of 10-16 animals for each of the four treatments.

cholic acid was not determined. Plasma was obtained prior to surgery, and cholesterol levels were determined by the method of Sperry and Webb (9).

Half-life and pool size were calculated as described by Lindstedt and Norman (10) by following decay of specific radioactivity (SR) with time and assuming that synthesis of nonlabeled bile acid causes the decay. The decay equation, $\frac{d(SR)}{dt} = -K\,(SR)$, integrates to $ln_e\,(SR) = -Kt + ln_e\,(SR_o)$, where K, the slope of a linear plot, is the fraction of the pool synthesized in unit time. Extrapolation of the plot, 2.303 log (SR) vs. t, to the ordinate gives a measure of SR_o or specific radioactivity before dilution by new synthesis. Pool size (mass) is then obtained from mass = $\frac{\text{injected label}}{SR_o}$, and synthesis rate = K x pool size is then calculated. Finally, from the relationship $t_{1/2} = \frac{0.69}{K}$ the half-life is calculated.

RESULTS AND DISCUSSION

Secretion of the two bile acids, cholate (C) plus chenodeoxycholate (CDC), averaged from 2.56 to 3.15 mg/15 min and was apparently not influenced by dietary treatment. However the ratio C/CDC was two- to three-fold higher in the diet A groups than in the diet B groups (Table I). Furthermore it was evident that in the absence of dietary cholate (diet B) dietary safflower oil tended to suppress CDC, in contrast with dietary beef tallow.

Specific radioactivity of cholate in the bile immediately after injection of label, obtained by extrapolation of the decay line (Fig. 1), was substantially lower in the SFO group than in

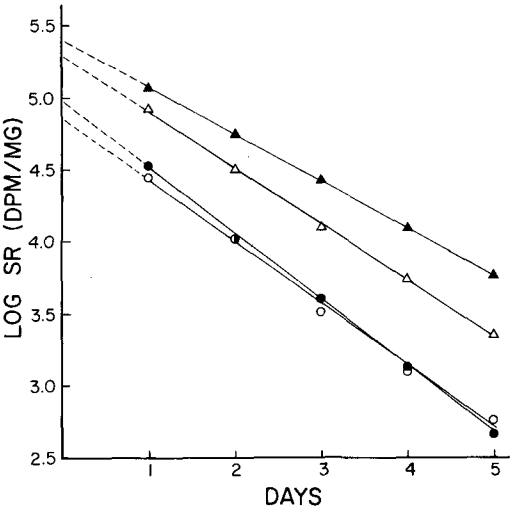

FIG. 1. Decline of cholate specific radioactivity (SR) as affected by diet. Rats were grown to 300 g on diet A with 5% SFO (○) or 5% BT (●), and diet B with 5% SFO (△) or 5% BT (▲), bile ducts were T-cannulated, and 3-4 days later ^{3}H-cholic acid was injected by cardiac puncture. Each point represents mean value from 15 min bile samples from 10-14 animals. Lines were computed by least squares method.

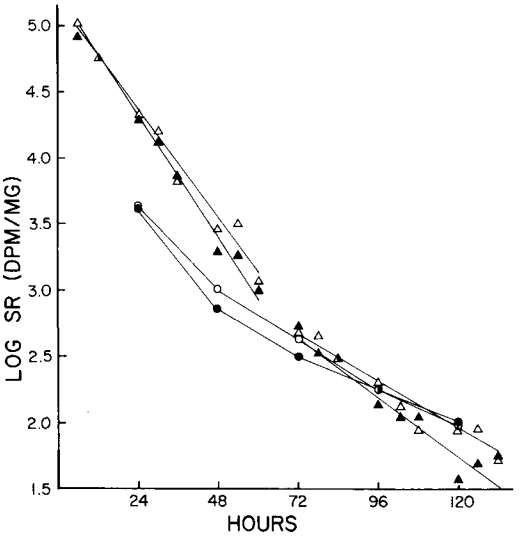

FIG. 2. Decline of chenodeoxycholate specific radioactivity (SR) as affected by diet. Legend of Figure 1 applies, except that [24-^{14}C] chenodeoxycholic acid was injected. Points for diet A series are mean values from six to eight animals; those for diet B series, two to six animals.

the BT group on both diets. This reflected a larger cholate pool size in the safflower-fed groups. In the absence of dietary cholate (diet B), the safflower-fed animals also had a more rapid cholate synthesis rate and a shorter cholate half-life; these results are consistent with a slightly higher rate of cholate secretion. The difference in cholic acid turnover constitutes a drain on the endogenous cholesterol and is consistent with the lower plasma cholesterol of the animals that consumed a polyunsaturated fat, safflower oil. The observation is also consistent with our earlier findings in a 6 hr period of bile collection that a safflower group had 13.6% higher conversion of cholesterol to bile acids than a beef tallow group (11). The results are also consistent with those reported by Gordon et al. (1), who observed a decreased half-life and increased synthesis rate of cholic acid in a human subject when the dietary fat was changed from butterfat to corn oil, and with those of Lindstedt et al. (2), who observed a shorter cholic acid half-life with humans fed corn oil as compared to coconut oil in a liquid formula diet. Although neither observed a difference in pool size, both reported a lower CDC percentage with dietary corn oil. Hellstrom and Lindstedt (12) also have reported that replacing butter with corn oil in a human diet increased the C/CDC ratio consistently and increased the cholate turnover rate in six of nine patients.

In contrast to the linear decline of specific radioactivity observed with cholic acid over the 5 day period, the specific radioactivity of chenodeoxycholic acid declined in a linear course for only 2-3 days, after which a different linear course was followed. The meaning of this double exponential type of decline is not clear. However it appears to be consistent, since it was observed also in our earlier studies with rats that were fed the colony diet (7). Because of this nonlinearity, meaningful turnover information is not obtainable from these isotope data. It is of interest, however, that the relatively lower CDC present in bile was associated with polyunsaturate feeding as safflower oil. Therefore there is a similarity to observations made with cholestyramine (13), since cholestyramine feeding has also been shown to increase the percentage of cholate with a simultaneous decrease in CDC. The difference, of course, is that cholestyramine usually decreases the cholate pool size, but the result appears to be the same: an increased cholate synthesis rate and a decreased cholate half-life with a consequent lower level of cholesterol in both plasma and liver.

Control animals in our experiments that were fed diets A and B but were not subjected to bile duct surgery or bile acid injection showed, at the termination of the experiment, plasma cholesterol levels of 150 and 246 mg/100 ml for SFO and beef tallow, respectively, in diet A, and 128 vs. 221 mg/100 ml in diet B. Hepatic cholesterol levels with diet B were 24 and 39 mg/g for SFO and BT, respectively. The experimental animals maintained a relatively good physiological state throughout the experiment. After some loss in body weight immediately following surgery they sustained only minor losses of 1-2% of body weight during the 5 day bile collection period, despite daily or more frequent ether anesthetization during collection periods.

The demonstration in this study, through direct collection of bile, that polyunsaturated fat in the diet stimulates cholate formation and shortens cholate half-life lends strong evidence to the view that the action of polyunsaturated fat in reducing levels of cholesterol in plasma and liver of the cholesterol-fed rat is through the stimulation of cholesterol oxidation.

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REFERENCES

1. Gordon, B.A., A. Kuksis and J.M.R. Beveridge, Can. J. Biochem. 42:897 (1964).
2. Lindstedt, S., J. Avigan, D.S. Goodman, J. Sjövall and D. Steinberg, J. Clin. Invest. 44:1754 (1965).
3. Hellstrom, K., J. Sjövall and G. Wigand, J. Lipid Res. 3:405 (1962).
4. Byers, S.C., and M. Friedman, Proc. Soc. Exp. Biol. Med. 98:523 (1958).
5. Lewis, B., T.R.E. Pilkington and K.A. Hodd, Clin. Sci. 20:249 (1961).
6. Wood, P.D.S., F. Aylward and T.R.E. Pilkington, Brit. J. Nutr. 17:89 (1963).
7. Klauda, H.C., R.F. McGovern and F.W. Quackenbush, Lipids 8:466 (1973).
8. Quackenbush, F.W., and M.D. Pawlowski, J. Nutr. 72:196 (1960).
9. Sperry, W.M., and M. Webb, J. Biol. Chem. 187:97 (1950).
10. Lindstedt, S., and A. Norman, Acta Physiol. Scand. 38:121 (1957).
11. McGovern, R.F., and F.W. Quackenbush, Lipids 8:473 (1973).
12. Hellstrom, K., and S. Lindstedt, Amer. J. Clin. Nutr. 18:46 (1966).
13. Wood, P., R. Shioda, D. Estrich and S. Splitter, Metab. Clin. Exp. 21:107 (1972).

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Effect of Dietary Fat Saturation on Absorption and Intestinal Secretion of Cholesterol by the Hypercholesterolemic Rat[1]

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ABSTRACT

The fate of an oral dose of [4-^{14}C] cholesterol given to rats grown on diets with 20% safflower oil or 20% hydrogenated coconut oil was determined by analysis of digestive tract, feces and tissues. The pattern of isotope distribution did not support the view that rats fed a saturated fat absorb less cholesterol than those fed an unsaturated fat. Fasted animals grown on the diet with 5% of these two fats and beef tallow showed no clear difference in the amount of digitonin-precipitable sterol in their intestines. A shorter transit time for intestinal contents was observed with the saturated fat groups. It is concluded that neither absorption of cholesterol from the gut nor secretion of β-hydroxy sterol into the gut accounts for the hypocholesterolemic effect of polyunsaturated fat.

INTRODUCTION

In efforts to explain the hypocholesterolemic effect of polyunsaturated fat it has been reported that unsaturated fat inhibits absorption of cholesterol from the gut (1,2). However others (3,4) have reported that unsaturated fats enhance absorption of cholesterol. In this laboratory thoracic lymph studies did not reveal a difference of cholesterol absorption from several unsaturated and saturated fats, except hydrogenated coconut oil, which showed reduced absorption (5).

In the present study we have employed intestinal transit and absorption experiments to observe the effects of unsaturation in dietary fat upon cholesterol absorption.

EXPERIMENTAL PROCEDURES

For the absorption study, two groups of weanling male Wistar strain rats were fed either 20% hydrogenated coconut oil (HCO) or 20% safflower oil (SFO) in a diet of the following per cent composition: glucose (Cerelose), 52.5; casein, 18.0; fat, 20.0; Celluflour, 2.0; cholesterol, 1.0; sodium glycocholate, 0.5; and minerals and vitamins as described previously (6), for a period of 10 weeks. Each was then given orally 0.5 μCi of [4-^{14}C] cholesterol (New England Nuclear, Boston) with 290 mg of the respective diet in a no. 3 gelatin capsule, with 15 min surveillance to assure complete ingestion before confinement in a metabolism cage. After 8 hr a blood sample was taken by heart puncture, and liver and intestines were removed. Cholesterol contents of the liver extract (7) and of the blood plasma were determined via the digitonide (8). The amount of radioactivity in the stomach and intestinal contents, liver, plasma, intestinal wall and urine was determined from appropriate extracts prepared as follows. Blood plasma was extracted with acetone-ethanol 1:1, and livers were extracted with chloroform-methanol (7). The intestinal contents were collected in 50 ml 0.9% saline and extracted three times with three volumes ether-hexane-ethanol 1:1:1, as described by Hofmann and Borgstrom (9). The washed intestines, stomach, and colon and feces were treated with 10 ml base (50 g potassium hydroxide to 100 ml water to 300 ml methanol) and 15 ml methanol-water 3:1, and heated at 80 C for 3 hr. After cooling, the digest was shaken with a mixture of 25 ml water, 30 ml ether and 30 ml pentane in a separatory funnel. The upper phase was extracted with 50% ethanol; the lower phase was extracted with ether-pentane 1:1 and this extract then washed with 50% ethanol. The ether-pentane extracts were then combined as neutral lipid extract. Portions of each of these different extracts were taken to dryness and dissolved in toluene scintillator (4.0 g/liter 2,5-diphenyloxazole and 0.1 g/liter 1,4-bis-2-[5-phenyloxazolyl]-benzene) for counting (Packard Tri Carb). Urine was evaporated to dryness in a scintillation vial and 1 ml of methanol added; then 15 ml toluene scintillator was added for counting. Quenching error was corrected by the channels ratio technique or by internal standards.

Animals to be used for intestinal secretion studies were fed 5% SFO, 5% beef tallow (BT) or 5% HCO in the same diet, adjusted isocalorically, for a period of 7 weeks. On the day preceding the experiment the animals were given 2 ml 5% aqueous polyethylene glycol as a nonabsorbable marker, and fasted for 24 hr.

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TABLE I

Summary of Data from Experiments on Absorption of [4-^{14}C] Cholesterol

Parameter	SFO	HCO
Dietary fat, %	20	20
No. of animals	10	9
Mean body wt, g	440	300
Plasma cholesterol, free, mg/100 ml	28	76
Plasma cholesterol, total, mg/100 ml	154	438
Hepatic cholesterol, free, mg/g	4.4	4.4
Hepatic cholesterol, total, mg/g	89.4	69.4
Plasma cholesterol per rat, mg[a]	24	46
Hepatic cholesterol per rat, g	2.84	1.39
Recovery of isotope, % of dose		
Intestinal contents and stomach	34.6 ± 6.1	18.5 ± 2.2[b]
Intestinal tissue	13.6 ± 2.0	11.8 ± 1.5
Colon and feces	14.7 ± 4.7	21.4 ± 2.5
Liver	8.2 ± 1.7	9.4 ± 1.5
Plasma[a]	3.4 ± 0.7	6.2 ± 0.7
Unabsorbed isotope, % of dose[c]	49.3	39.9

[a]Blood plasma volume was assumed to be 3.5% body wt.

[b]Mean ± SEM.

[c]Consists of isotope recovered in stomach, intestinal contents, colon and contents, and feces.

The bile flow was diverted by external cannula, and after 6 hr the intestines were removed and the contents collected in 50 ml 0.9% saline and extracted three times with 50 ml portions of ether. Sterol in the ether extract was determined via the digitonide; the aqueous phase was then analyzed for polyethylene glycol by the following method. Ten milliliters of the solution was placed in a 25 ml erlenmeyer flask; 1 ml polyethylene glycol standard was placed in a second flask; and 1 ml water was placed in a third flask. Water was added to all flasks to a total of 11 ml, and 1.0 ml 10% barium chloride, 2.0 ml 0.30 N barium hydroxide and 2.0 ml of 5% zinc sulfate were added to each. The mixtures were shaken and after 10 min were filtered through Whatman No. 42 filter paper. One milliliter of gum arabic was added with mixing followed by 4.0 ml 30% trichloroacetic acid containing 5% barium chloride. The tubes were shaken, and after exactly 1 hr absorbance was determined against a reagent blank at 650 mμ and polyethylene glycol values were calculated.

RESULTS AND DISCUSSION

At 10 weeks animals fed 20% safflower oil had a much lower level of both free and total plasma cholesterol than those fed 20% hydrogenated coconut oil (Table I). Hepatic cholesterol levels of the SFO animals, on the other hand, were substantially higher than those of HCO animals; the total amount per rat was more than doubled. The difference can be attributed in part to the differences in body weight, but the data suggest that polyunsaturated fat at the 20% level tends to increase hepatic cholesterol ester accumulation while lowering plasma cholesterol. These data are consistent with results obtained earlier with the same basal diet, in which soybean oil was compared with hydrogenated coconut oil at the 20% level (6).

When [4-^{14}C] cholesterol was administered to such animals, differences were also seen in the distribution of the isotope in the digestive tract and in tissues. Isotope recovery in the stomach and its contents, intestinal contents, the colon and its contents, and feces from the SFO-fed animals totaled 49.3% of the administered dose as compared to 39.9% for the HCO-fed animals. These results suggest a slower movement through the digestive tract of the SFO-fed rats. In an 8 hr period it is to be expected that much of the ingesta remaining in the small intestine will be distal to the primary site of absorption (10). The urine from both groups contained only a trace of isotope; total carcass was not analyzed. However the sum of isotope recovery from intestinal tissue, liver and plasma should provide an index of the relative amounts absorbed. These values do not differ significantly for the two groups and one must therefore conclude that the difference in digitonin-precipitable sterol accumulation between animals fed unsaturated fat (SFO), and saturated fat (HCO) does not reflect a difference in the amount of cholesterol absorbed. This evidence is at variance with the conclusions of Byers and Friedman (3), who analyzed thoracic lymph from rats that had ingested cholesterol

TABLE II

Influence of Dietary Fat on Intestinal Nonbiliary Digitonin-Precipitable Sterol and Intestinal Motility[a]

Dietary fat	Body wt, g	Intestinal wt, g	Sterol, mg	Polyethylene glycol, mg
SFO	317	7.55	3.82 (8)	5.11
HCO	250	6.99	3.29 (9)	1.31
BT	303	7.68	2.95 (9)	0.86

[a]Rats were fed the 5% fat diets for 7 weeks, then given by gastric intubation 100 mg polyethylene glycol; after 24 hr their bile ducts were cannulated to flow externally. Six hours later the intestinal contents, exclusive of stomach and colon, were collected in 50 ml 0.9% saline and analyzed. Details are in text. Numbers in parentheses represent number of animals.

in different fats and found that more cholesterol was absorbed from corn oil or soybean oil than from lard or hydrogenated coconut oil. Klauda and Quackenbush (5) concluded from lymph composition studies that, while hydrogenated coconut oil represents a special case because of its high content of short-chained acids, there were no significant differences in cholesterol absorption from a number of other saturated and unsaturated fats. Thus in this laboratory we arrive at the same conclusion from two different types of experimentation: that polyunsaturated fats do not substantially affect cholesterol absorption from the gut.

The question arose as to whether dietary polyunsaturated fat may in some way facilitate reverse absorption, i.e., promote secretion of sterol into the intestine. In the experiment to investigate this (Table II), polyethylene glycol was used as a nonabsorbable marker. The results showed a slightly higher digitonin-precipitable sterol content in the group grown on dietary SFO than in those groups grown on saturated fat diets. However the SFO group also had a substantially higher residual polyethylene glycol in the intestine. It is therefore reasonable that with lower intestinal motility and less rapid peristaltic movement a larger amount of sterol was allowed to accumulate, even though the rate of secretion into the gut may have been the same in all groups. Accordingly, one must conclude that no large difference exists in the rate of sterol secretion into the intestine as a consequence of degree of unsaturation of dietary fat.

From these experiments it does not appear that the plasma cholesterol lowering effect of polyunsaturated fats can be explained through an influence upon either the absorption of cholesterol from, or secretion into, the intestine. The data do suggest that, at the higher levels of dietary fat intake, a polyunsaturated fat may influence the partition of cholesterol between plasma and liver.

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F. Castor performed the bile duct cannulations. Contributions of materials were beef tallow by Swift & Co., hydrogenated coconut oil by Procter & Gamble, and safflower oil by Pacific Vegetable Oil Corp. This work was supported in part by U.S. Public Health Service Grant 5-Tol GM 1195.

REFERENCES

1. Wood, P., R. Shioda and L. Kinsell, Lancet 2:604 (1966).
2. Wood, P., Y. Lee and L. Kinsell, Fed. Proc. 26:261 (1967).
3. Byers, S., and M. Friedman, Proc. Soc. Exp. Biol. Med. 98:523 (1958).
4. Bloomfield, D., J. Lab. Clin. Med. 64:613 (1964).
5. Klauda, H., and F. Quackenbush, Lipids 5:142 (1970).
6. Quackenbush, F.W., and M. Pawlowski, J. Nutr. 72:196 (1960).
7. Harris, R., and D. Gambal, Anal. Biochem. 5:479 (1963).
8. Sperry, W.M., and M. Webb, J. Biol. Chem. 187:97 (1950).
9. Hofmann, A., and B. Borgstrom, J. Clin. Invest. 43:247 (1964).
10. Borgstrom, B., Ibid. 39:809 (1960).

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Influence of Dietary Fat on Bile Acid Secretion of Rats after Portal Injection of ^{3}H-Cholesterol and [4-^{14}C] Cholesteryl Esters[1]

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ABSTRACT

Labeled cholesterol and its esters were injected via the portal vein into bile duct-cannulated rats, subsequent to a 7 week regimen of either 5% safflower oil or 5% beef tallow in a hypercholesterolemic diet. Analysis of bile collected over a 6 hr period from the safflower group showed 8.6% higher output of bile acids, 13.6% higher conversion of ^{3}H-cholesterol to bile acids and 40% higher conversion of [4-^{14}C] cholesteryl oleate to bile acids than bile collected from the tallow group. During the lst hr conversion of both oleyl and linoleyl esters of ^{14}C-cholesterol to bile acids was much slower than conversion of the free ^{3}H-cholesterol to bile acids, thus eliminating these esters as preferred substrate for bile acid formation. However at 6 hr two-thirds of the injected ^{14}C of oleyl ester was recovered in the liver, and about half of this was in the form of free cholesterol. Thus cholesterol ester hydrolase, though inhibited by dietary cholesterol, evidently did not impose limitations on formation of free cholesterol for subsequent oxidation reactions. Specific radioactivities were of doubtful significance because of uncertainities as to "active" pool size. The data suggest that dietary linoleate exerts its hypocholesterolemic effect in some manner other than ester formation and that its point of action involves stimulation of cholesterol oxidation to bile acids.

INTRODUCTION

Although it is well established that polyunsaturated fats can lower plasma cholesterol levels, the fate of the cholesterol that is removed from the plasma compartment has not been clarified. Simple redistribution to other tissues does not account for the differences observed with saturated and unsaturated dietary regimens (1). In the rat it is hypothesized that excretion of neutral sterols or bile acids is enhanced by unsaturated fat feeding (2,3). Although differences in the rate of oxidation of cholesterol by hepatic mitochondria have been reported (4), studies in which livers were perfused did not demonstrate a quantitative difference in bile acid production between the saturated and unsaturated fat-fed animals (5). On the other hand, ^{14}C-cholesterol of lipoproteins produced from mevalonate in vivo by rats fed soybean oil was eliminated as bile acids more rapidly than that produced in rats fed butterfat when the lipoproteins were injected into normal rats (6). Those authors suggested that unsaturation in the cholesteryl esters of the lipoproteins could be responsible for these differences.

In the present study we have used safflower oil-fed rats and beef tallow-fed rats that had established their different patterns of plasma and tissue cholesterol levels in response to cholesterol feeding, and we have observed that injected cholesterol and cholesteryl esters are secreted as bile acids more rapidly by the safflower oil-fed rats.

EXPERIMENTAL PROCEDURES

Male, weanling, Wistar rats were fed a semipurified diet (1), which contained 5% of either safflower oil (SFO, Pacific Vegetable Oil Corp., Richmond, Calif.) or beef tallow (BT, Swift & Co., Chicago) for a period of 7 weeks. The animals were fasted overnight, and bile ducts were cannulated with polyethylene tubing (PE-10) in the upper part of the bile duct. As soon as flow was established, the radioisotopic cholesterol in the form of a micellar preparation was injected via the portal vein.

The micellar preparations contained 2.4 μmol sodium taurocholate, 0.6 μmol oleic acid (Hormel Institute) and 0.3 μmol monoolein per milliliter calcium- and magnesium-free Krebs-Ringer phosphate buffer, pH 6.3. To this mixture was added, per milliliter, either (a) 8.55 x 10^{6} dpm [4-^{14}C] cholesteryl oleate (New England Nuclear, 50 mCi/mM) and 28.3 x 10^{6} dpm ^{3}H-cholesterol (Amersham-Searle, 672 mCi/mM); or (b) 10.50 x 10^{6} dpm cholesteryl linoleate (Amersham-Searle, 21 mCi/mM). The

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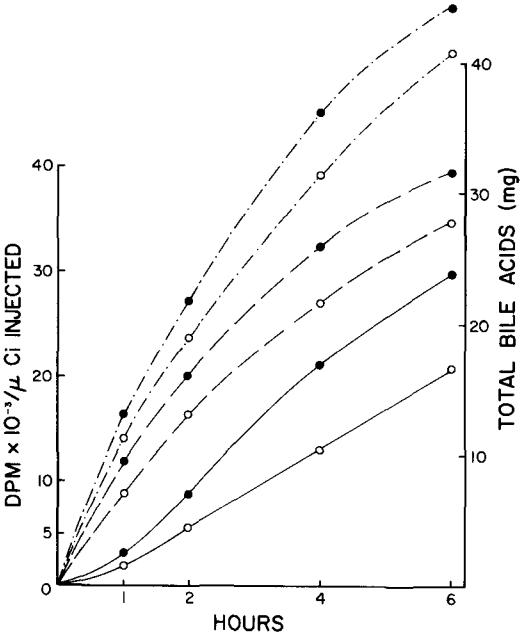

FIG. 1. Cumulative secretion after bile duct surgery and portal injection of ^{3}H-cholesterol and [4-^{14}C] cholesteryl oleate. Rats were fed a semi-purified diet containing 5% of SFO (closed circles) or 5% of BT (open circles) for 7 weeks, then subjected to bile duct surgery. When bile began to flow from the cannula, 0.20 ml micellar preparation containing 2.55 μCi ^{3}H-cholesterol and 0.77 μCi [4-^{14}C] cholesteryl oleate was injected into the portal vein. Bile samples were collected continuously as four fractions and analyzed for total bile acids, —·—·—: ^{3}H-radioactivity of bile acids, — — —; and ^{14}C-radioactivity of bile acids, ——. Points represent mean values for 8-12 animals.

mixtures were sonicated until transparent. The micellar cholesteryl oleate was prepared in quantity and used over a period of days, during which time some turbidity developed as observed by Thompson et al. (7). The micellar cholesteryl linoleate was prepared daily as needed and was clear when injected; it was probably a superior dispersion for our experimentation. Radiochemical purity of the ^{3}H-cholesterol and the [4-^{14}C] cholesteryl esters was 95+% by thin layer chromatographic (TLC) analysis. Sodium taurocholate was prepared by the procedure of Norman (8), and monoolein was isolated from a lipase reaction mixture (9).

Bile samples were collected continuously after injection of radioisotope, as four fractions at 0-1, 1-2, 2-4 and 4-6 hr. After the 6 hr collection period a blood sample was taken by cardiac puncture and the liver was then removed. Plasma and liver were analyzed for free and total cholesterol via the digitonide (1). Portions of the extracts were subjected to analysis for total radioactivity and for radioactivity of the digitonin-precipitable sterol (free cholesterol).

Total bile acids were determined by the method of Levin et al. (10) with the modification that polyethylene tubes were used in saponifying the samples. Absorbance was found to be linear for concentrations of bile acid from 5 to 30 μg, and all samples were diluted to read within this range. Radioactivity in the bile acid fraction was determined by adding 1 ml ethanol to the bile acid residue, followed by 15 ml toluene scintillator and counting in a Packard Tri Carb Spectrometer. Quenching was corrected by the channels ratio method or by internal standards.

Cholesteryl ester hydrolase activity was determined by the method of Deykin and Goodman (11). However for complete removal of endogenous substrate the 100,000 x g supernate was dispersed in hexane in a test tube with a loose fitting Teflon pestle. When the phases separated endogenous ester was removed in the hexane phase, and at least 60% of the enzyme activity was recovered in the aqueous phase. Radioactive cholesteryl oleate, synthesized according to the method of Pinter et al. (12) and purified by TLC on Silica Gel G with hexane-ether-acetic acid 70:30:1, was added in acetone (130 mμmol ester per 100 μl) to 2.0 ml aqueous enzyme preparation. After incubation at 37 C for 1 hr, 50 ml chloroform-methanol 2:1 v/v was added to stop the reaction and extract the cholesterol. The next morning 10

TABLE I

Cholesterol Levels in Liver and Plasma of Animals Fed Either 5% SFO or 5% BT[a]

Dietary fat	Liver, mg/g		Plasma, mg/100 ml	
	Total	Free	Total	Free
5% SFO	75.3 ± 3.4(9)	2.46 ± 0.12(9)	188 ± 28(9)	33 ± 5(9)
5% BT	94.1 ± 4.2(11)	2.64 ± 0.06(11)	287 ± 46(10)	54 ± 9(10)

[a]Blood samples were taken by heart puncture at conclusion of bile collections 6 hr after cannulation. Animals were then killed and livers removed. Data are mean concentrations ± SEM; numbers in parentheses represent number of animals.

TABLE II

Fatty Acid Composition of Cholesterol Esters Isolated from Livers of Rats Fed Either 5% SFO or 5% BT

	Fatty acid carbon no., area %[a]						
Diet	14:0	16:0	16:1	18:0	18:1	18:2	20:4
SFO	Trace	9.1	12.9	2.8	40.6	29.4	5.2
BT	0.7	12.4	14.2	1.9	69.1	1.6	ND

[a]Values represent the analysis of a 10-animal pool, expressed as percentage of the total measurable fatty acids designated by chain length and number of double bonds; ND, not detected.

ml distilled water was added, and the chloroform layer was removed and evaporated under reduced pressure; 1 mg each of cholesterol and cholesteryl palmitate were added and the entire lipid extract applied to a Silica Gel G plate for separation of cholesterol from its esters with hexane-ether-acetic acid 90:20:1 v/v/v. Plates were air-dried and exposed to iodine vapors. The bands were scraped off and eluted with 20 ml chloroform-methanol 2:1 v/v. Toluene scintillator was added to the evaporated extract and radioactivity measured. Protein was determined by the method of Lowry et al. (15).

RESULTS AND DISCUSSION

Conversion of Free Cholesterol and Cholesteryl Oleate to Bile Acids

Total bile acid output was consistently higher during the 1st hr than during subsequent hours of the 6 hr period following cannulation (Fig. 1). A 6 hr period was selected because physiological control of bile acid synthesis is still exerted during this period (13,14). The cumulative output of bile acids during the 6 hr collection period was 8.6% higher in the SFO group than in the BT group. Secretion of sterol followed similar trends, but the quantity was less than 1% of the bile acids for the 6 hr period (0.156 mg for SFO and 0.136 mg for BT). Accordingly, no measurements were made of the sterol radioactivity.

Radioactivity in the bile acids arising from ^{3}H-cholesterol injection was 13.6% higher in the SFO group than in the BT group, based on per cent recovery of the injection dose. Total recovery at 6 hr was nearly 2% of the injected dose.

Secretion of ^{14}C as bile acids from injected [4-^{14}C]-cholesteryl oleate was 40% greater in the SFO group than in the BT group during the 6 hr period. The pattern of secretion from the ester was quite different from that of the free cholesterol. During the 1st hr, secretion of bile acids from the ester was only one-fourth that of secretion from free cholesterol. This concurs with the observations of Ogura et al. (16) from 3 hr collections of bile after injection of emulsions of labeled cholesterol and cholesteryl oleate into normal rats. The lag in conversion from the ester was apparently confined largely to the 1st hr; thereafter the curves took form similar to those observed with free cholesterol injections. It is reasonable that this lag represents a temporary delay in conversion of the ester to a suitable form for oxidation, presumably hydrolysis to free cholesterol.

The consistently higher rates of secretion by SFO-fed rats strongly supports the view that cholesterol-lowering by polyunsaturated fat feeding is the result of stimulated conversion of

TABLE III

Cholesteryl Ester Hydrolase Activity of Rat Liver as Influenced by Diet

Basal diet	Cholesterol, %	Bile salt, %	Hydrolase activity	
			Experiment I[a]	Experiment II[b]
5% SFO	1.0	0.5	314 ± 21(4)	
5% BT	1.0	0.5	288 ± 20(4)	
Colony			631 ± 20(3)	570(4)
Colony	1.0		323 ± 20(3)	368(4)
Colony	1.0	0.5	231 ± 14(3)	
Colony		0.5		564(4)

[a]Expressed as mμmoles cholesteryl oleate hydrolyzed per hour per gram protein; mean values ± SEM; number of animals in parentheses.

[b]Liver pooled in pairs before analysis; data are mean of two values.

TABLE IV

Distribution of Cholesterol Radioactivity 6 Hours after a Portal Injection of [4-^{14}C]Cholesteryl Oleate

Dietary fat	Liver			Plasma total, dpm/ml x 10^{-4}
	Total, dpm x 10^{-6}	Free		
		dpm x 10^{-6}	% of injected dose	
SFO	1.14	0.53	30.8	1.14
BT	1.13	0.52	30.6	1.28

cholesterol to bile acids. It is consistent with the often repeated demonstration that, in rats on hypercholesterolemic diets, cholesterol of both liver and plasma is lower when an adequate amount of unsaturated fat is fed (Table I). The fatty acid distribution in the cholesteryl esters of the SFO and BT rats is also typical of animals that receive these fats in the dietary. (Table II).

Cholesteryl Ester Hydrolase (CEH) Activity

The advantage shown for the metabolism of free cholesterol over that of oleyl ester in the lag period of Figure 1 suggests that cholesteryl ester hydrolase (CEH) activity might be of importance in bile acid formation. Quarfordt and Goodman (17) injected chylomicrons containing labeled cholesteryl esters into normal rats and observed that the cholesteryl esters were hydrolyzed in the liver to the extent of 60% in the 1st hr and 85-90% in 3.5 hr. They also observed distribution into various tissues but did not examine conversion to bile acids. In our experiments cholesteryl oleate was hydrolyzed less rapidly than in theirs, and the question arose whether this might reflect a stress from cholesterol and bile salt feeding. When we then fed colony diet in which cholesterol and bile salts were added, a ca. 50% reduction of the CEH activity of livers resulted (Table III). In experiment II it was shown that dietary cholesterol is largely responsible for this inhibition, since bile salt alone had no effect. There were no meaningful differences in CEH activity between the SFO and BT groups, either on the unit basis as shown or in the whole liver, since hepatic weights were equal.

Consideration was given to the possible importance of a reduced CEH activity in the overall metabolic process. The cholesteryl ester levels after 7 weeks on the SFO and BT diets were 73 and 92 mg per gram liver, respectively, while the corresponding free cholesterol values were only 3.3 and 2.8% of the total (Table I). Based on our earlier studies, we believe these are essentially steady-state conditions (1). If CEH activity were a limiting factor for cholesterol availability as substrate in the oxidation process, this should be reflected in the ester-free ratio of labeled cholesterol. Analysis of livers from [4-^{14}C]cholesteryl oleate-injected rats at the end of the 6 hr period revealed extensive hydrolysis of the esters (Table IV). More than 30% of the injected dose and more than 46% of the then remaining [4-^{14}C]cholesterol was in the free form. At this point 67% of the injected dose was recovered in the liver. Thus, despite the inhibitory effect of dietary cholesterol, the CEH was abundantly able to split injected ester. Furthermore the injected

TABLE V

Summary of Data Collected after Portal Injection of Oleyl and Linoleyl Esters of [4-^{14}C]Cholesterol[a]

Parameter	Oleyl ester		Linoleyl ester	
	SFO	BT	SFO	BT
0-1 hr bile acids, dpm/mg	185	140	528	369
1-2 hr bile acids, dpm/mg	492	373	1418	937
2-4 hr bile acids, dpm/mg	672	458	1449	1222
4-6 hr bile acids, dpm/mg	808	642	1273	1173
Hepatic ester, dpm/mg	632	500	758	484
Liver wt, g	13.3	13.2	12.9	11.1
Hepatic ester, mg/g liver	73	92	65	101

[a]Male rats were fed 5% SFO or BT for 7 weeks, then cannulated and injected with ca. 1 μCi of the ester. Bile was collected for 6 hr; then livers were taken for analysis. Hourly data are mean values of bile acids from bile from 8-12 animals per group.

cholesteryl ester was apparently hydrolyzed preferentially, i.e., the ester-free ratio (54:46) showed no resemblance to that in the liver as a whole (97:3). It is therefore apparent that the injected cholesteryl oleate entered a small metabolic pool that was readily hydrolyzed by CEH and within 6 hr was nearly half converted to the free form. If all of the free cholesterol in the liver were compartmentalized in this "active" pool at 6 hr, 2.46/.46 or 5.4 mg per gram of liver for the SFO group, and 2.64/.46 or 5.7 for the BT group, would constitute this metabolic pool, of which less than 3 mg was ester in each case; and more than 95% of the cholesteryl ester was in a different compartment and was not accessible to action of the hydrolase. It follows that, with 46% conversion to the free form and less than 2% oxidation to bile acids during the 6 hr period, there is only a remote possibility that CEH conversion of the ester to the free form is a rate-limiting step in the chain of reactions that converts cholesterol oleate to bile acids. The form of the accumulation curves (Fig. 1) further attests to this view.

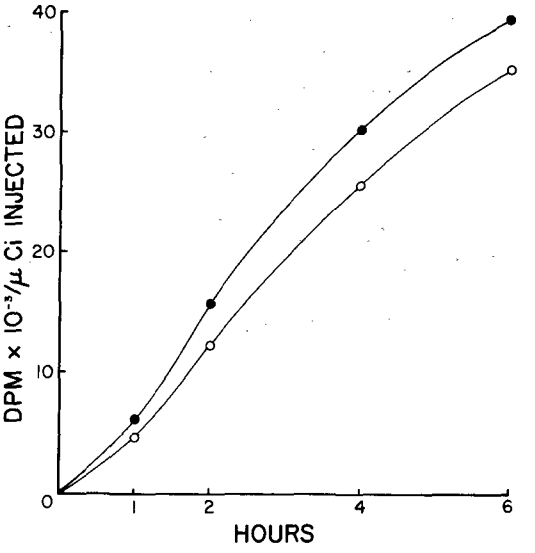

FIG. 2. Cumulative secretion of ^{14}C in bile acids after portal injection of [4-^{14}C] cholesteryl linoleate. Procedure as in Figure 1, except that micellar preparation containing 0.95 μCi labeled ester per 0.2 ml was prepared immediately before injection. SFO-fed rats, closed circles; BT-fed rats, open circles.

Conversion of Cholesteryl Linoleate to Bile Acids

The indication that cholesterol as oleyl ester converts to bile acids less readily than free cholesterol does not assure that free cholesterol is the primary substrate for oxidation. Ogura et al. (16) also emphasized this point. Boyd (18) has suggested that linoleate ester may be the metabolite for 7-α-hydroxy cholesterol formation. Swell and Law (19,20) injected a Tween 20 suspension of [4-^{14}C] cholesteryl esters and observed more rapid metabolism of linoleic and arachidonic esters than of palmitic or oleic. Since the linoleate level of cholesteryl esters of SFO-fed rats contrasts sharply with that of BT-fed rats (Table II), bile acid production from [4-^{14}C] cholesteryl linoleate is of interest (Fig. 1). As with cholesteryl oleate injections, there was a lag in the conversion of the labeled cholesterol into bile acids. Again, the lag was essentially confined to the 1st hr, and contrasted sharply with the rapid early conversion of free cholesterol. It is apparent that neither of these esters is a primary substrate for cholesterol oxidation to bile acids.

Specific activities of the bile salts are shown in Table V. The higher values for those derived from cholesteryl linoleate reflect a larger injected dose of radioisotope and probably a superior physical state of the micellar preparation at the time of injection. Because of the obvious compartmentalization of the cholesterol in the liver as a whole, it is not possible to relate with any certainty these bile acid radioactivities to those of esters in the liver at the end of the 6 hr period. If one chooses to make calculations based on total cholesterol esters as the pool size and to assume that CEH activity is a limiting factor with no preference for oleate or linoleate as a substrate (13), a different result is deduced than if one assumes a much smaller active pool size and no practical limitations of CEH activity in providing substrate for oxidation. Whatever the correct assumption may be, it is apparent that the advantages of SFO-fed rats in converting cholesterol to bile acids are attributable to other (subsequent?) factors than those examined in this study.

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REFERENCES

1. McGovern, R.F., and F.W. Quackenbush, Lipids 8:470 (1973).
2. Byers, S.O., and M. Friedman, Proc. Soc. Exp. Biol. Med. 98:523 (1958).
3. Wilson, J.D., and M.D. Siperstein, Amer. J. Physiol. 196:596 (1959).
4. Kritchevsky, D., R.R. Kolman, M.W. Whitehouse, M.C. Cottrell and E. Staple, J. Lipid Res. 1:83 (1959).

5. Klevay, L.M., and D.M. Hegsted, J. Atheroscler. Res. 8:329 (1968).
6. Lewis, B., T.R.E. Pilkington and K.A. Hodd, Clin. Sci. 20:249 (1961).
7. Thompson, G.R., R.K. Ockner and K.J. Isselbacher, J. Clin. Invest. 48:87 (1969).
8. Norman, A., Arkivi Kemi 8:331 (1955).
9. Mattson, F.H., and R.A. Volpenhein, J. Lipid Res. 2:58 (1961).
10. Levin, S.J., J.L. Irvin and C.G. Johnston, Anal. Chem. 33:856 (1961).
11. Deykin, D., and D.S. Goodman, J. Biol. Chem. 237:3649 (1962).
12. Pinter, K.G., J.G. Hamilton and J.E. Muldrey, J. Lipid Res. 5:273 (1964).
13. Myant, N.B., and H.A. Eder, Ibid. 2:363 (1961).
14. Shefer, S., S. Hauser, I. Bekersky and E.H. Mosbach, Ibid. 10:646 (1969).
15. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193:265 (1951).
16. Ogura, M., J. Shiga and K. Yamasaki, J. Biochem. (Tokyo) 70:967 (1971).
17. Quarfordt, S.H., and D.S. Goodman, J. Lipid Res. 8:264 (1967).
18. Boyd, G.S., Fed. Proc. 21:86 (1962).
19. Swell, L., and M.D. Law, Arch. Biochem. Biophys. 112:115 (1965).
20. Swell, L., and M.D. Law, Ibid. 113:143 (1966).

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SHORT COMMUNICATIONS

Separation of *cis* and *trans*-Isomers of 1,2-Benzylidene Glyceryl Esters

ABSTRACT

Separation of the *cis*- and *trans*-isomers of 1,2-benzylidene glyceryl esters has not yet been achieved by column or gas liquid chromatography. However these isomers were separated by thin layer chromatography in the present study and the parent cyclic alcohols regenerated by alkaline hydrolysis.

As part of a program to study the mass spectra of benzylidene and isopropylidene derivatives of monoglycerides, the chromatographic separation of these compounds was investigated. 1,2-Isopropylidene glycerol is the only product of the reaction of glycerol and acetone (1), but both 1,2- and 1,3-benzylidene glycerol are produced in the corresponding reaction with benzaldehyde (1-5). Furthermore each of the products of the latter reaction is obtained in the *cis* and *trans* forms (with differing relative positons of the phenyl and hydroxymethyl or hydroxy groups). Esters of isopropylidene glycerol and of 1,3-benzylidene glycerol are used as intermediates in the preparation of monoglycerides (6-8). The separation of 1,2-benzylidene glyceryl palmitate and 1,3-benzylidene glyceryl palmitate by crystallization or by column chromatography is possible, and the latter compound can be further separated chromatographically into its *cis* and *trans* forms (4). The complete separation of *cis*- and *trans*-1,2-benzylidene glyceryl esters or of their parent alcohols has not been reported previously. This paper describes a method for the separation of *cis*- and *trans*-1,2-benzylidene glyceryl esters by thin layer chromatography (TLC) with subsequent regeneration of the parent cyclic alcohols by hydrolysis.

An isomeric mixture of benzylidene glycerols, prepared by the acid-catalyzed condensation of glycerol and benzaldehyde, was esterified with palmitoyl chloride in the presence of pyridine (8). The esters were separated into four bands (Table I) by preparative TLC with double development, and the esters were eluted with diethyl ether. The NMR spectra of the compounds dissolved in either deuterochloroform or carbon tetrachloride from the bands with R_f values of 0.58 and 0.38 corresponded to those previously recorded for *trans*- and *cis*-1,3-benzylidene glyceryl palmitate, respectively (5). NMR spectra of the compounds eluted from the central bands indicated that each compound was a pure isomer of 1,2-benzylidene glyceryl palmitate (4).

For the recovery of the cyclic alcohols, 1.0 ml methanol containing 30 mg ester and 10 mg potassium hydroxide was heated at 50 C for 10 min. Carbon dioxide was then bubbled through the reaction mixture until the solution was acid to phenolphthalein. Ten milliliters carbon tetrachloride was added, the solution filtered and the solvents removed under reduced pressure. The resulting alcohols were purified by preparative TLC using petroleum ether-diethyl ether 25:75 v/v as solvent. The plates were developed twice and the alcohols, as oils, were eluted with diethyl ether. Absence of a peak at 5.87 ppm in the NMR spectrum of the *trans*-isomer and of a peak at 5.72 ppm in the spectrum of the

TABLE I

TLC and NMR Data for Benzylidene Glyceryl Palmitates[a]

Compounds identified	TLC R_f values[b]	NMR benzyl hydrogen absorption, ppm[c]
1,3-*trans*-isomer	0.58	5.33
1,3-*cis*-isomer	0.38	5.53
1,2-*trans*-isomer	0.45	5.72
1,2-*cis*-isomer	0.50	5.87

[a]TLC = thin layer chromatography.

[b]Solvent system petroleum ether-diethyl ether 75:25 v/v.

[c]δ scale.

cis-isomer indicated that the preparations were the two pure isomers of 1,2-benzylidene glycerol.

The study was extended to include the acetate and benzoate esters of the benzylidene glycerols. These esters were also separated into four isomers each by preparative TLC, and results similar to those described above were obtained. Due to the higher polarity of the acetate esters, a greater proportion of diethyl ether was required in the TLC solvent system than was required for the other esters. Unesterified benzylidene glycerols have been partially separated by column and gas liquid chromatography (5). TLC separation of the benzylidene glycerols prepared by the acid-catalyzed condensation of glycerol and benzaldehyde in the present study yielded three bands with R_f values of 0.57, 0.40 and 0.28. NMR spectra of the fractions eluted from these bands showed, by comparison with previously recorded spectra (4), that the fastest and slowest moving bands were *trans*- and *cis*-1,3-benzylidene glycerol, respectively, and that the central band was a mixture of *cis*- and *trans*-1,2-benzylidene glycerol. Therefore the dioxolane isomers of benzylidene glycerol could not be separated directly by TLC with the conditions used in this study. However the four benzylidene glycerol isomers can be prepared in a pure state by the esterification-TLC separation-hydrolysis procedure as described above.

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REFERENCES

1. Showler, A.J., and P.A. Darley, Chem. Revs. 67:427 (1967).
2. Baggett, N., J.S. Brimacombe, A.B. Foster, M. Stacey and D.H. Whiffen, J. Chem. Soc. 1960:2574.
3. Baggett N., J.M. Duxburg, A.B. Foster and J.M. Webber, Ibid. (C) 1966:208.
4. Serdarevich, B., and K.K. Carroll, Can. J. Biochem. 44:743 (1966).
5. Serdarevich, B., JAOCS 44:381 (1967).
6. Malkin, T., and T.H. Bevan, in "Progress in the Chemistry of Fats and Other Lipids," Vol. 4, Edited by R.T. Holman, W.O. Lundberg and T. Malkin, Pergamon Press, New York, 1957, p. 63.
7. Hartman, L., Chem. Revs. 58:845 (1958).
8. Mattson, F.H., and R.A. Volpenhein, J. Lipid Res. 3:281 (1962).

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Identification of Some Polyenoic Acids Isolated from Human Testicular Tissue

ABSTRACT

Five polyenoic acids present in human testicular tissue have been isolated in pure form by gas chromatography and chemically identified using procedures of hydrogenation and UV absorption spectrometry following alkaline isomerization and ozonolysis. Three of these (20:3, 22:4 and 22:5) belong to the linoleic acid and the other two (22:6 and 22:5), to the α-linolenic acid family. The latter (22:5ω3) had not been reported previously in human testes; the others had been tentatively identified by retention time using gas chromatography.

INTRODUCTION

Testicular fatty acids of several species have been well characterized (1-3), but little information is available for fatty acids of human testes. Polyenoic acids of 20 and 22 carbons chain length comprise a major portion of testicular fatty acids of most species, including man. Some of these have been identified and quantitated in human tissue by Bieri and Prival (4). However identification of the polyenes by these investigators was solely by gas chromatographic assay. In order to confirm the identity of the polyenoic acids of human testicular tissue, it is important to characterize them by chemical means. In this paper we report the gas chromatographic and chemical characterization of a number of polyenoic acids isolated in pure form from human testicular tissue.

EXPERIMENTAL PROCEDURES

Human testicular tissue was obtained at

cis-isomer indicated that the preparations were the two pure isomers of 1,2-benzylidene glycerol.

The study was extended to include the acetate and benzoate esters of the benzylidene glycerols. These esters were also separated into four isomers each by preparative TLC, and results similar to those described above were obtained. Due to the higher polarity of the acetate esters, a greater proportion of diethyl ether was required in the TLC solvent system than was required for the other esters. Unesterified benzylidene glycerols have been partially separated by column and gas liquid chromatography (5). TLC separation of the benzylidene glycerols prepared by the acid-catalyzed condensation of glycerol and benzaldehyde in the present study yielded three bands with R_f values of 0.57, 0.40 and 0.28. NMR spectra of the fractions eluted from these bands showed, by comparison with previously recorded spectra (4), that the fastest and slowest moving bands were *trans*- and *cis*-1,3-benzylidene glycerol, respectively, and that the central band was a mixture of *cis*- and *trans*-1,2-benzylidene glycerol. Therefore the dioxolane isomers of benzylidene glycerol could not be separated directly by TLC with the conditions used in this study. However the four benzylidene glycerol isomers can be prepared in a pure state by the esterification-TLC separation-hydrolysis procedure as described above.

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REFERENCES

1. Showler, A.J., and P.A. Darley, Chem. Revs. 67:427 (1967).
2. Baggett, N., J.S. Brimacombe, A.B. Foster, M. Stacey and D.H. Whiffen, J. Chem. Soc. 1960:2574.
3. Baggett N., J.M. Duxburg, A.B. Foster and J.M. Webber, Ibid. (C) 1966:208.
4. Serdarevich, B., and K.K. Carroll, Can. J. Biochem. 44:743 (1966).
5. Serdarevich, B., JAOCS 44:381 (1967).
6. Malkin, T., and T.H. Bevan, in "Progress in the Chemistry of Fats and Other Lipids," Vol. 4, Edited by R.T. Holman, W.O. Lundberg and T. Malkin, Pergamon Press, New York, 1957, p. 63.
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INTRODUCTION

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EXPERIMENTAL PROCEDURES

Human testicular tissue was obtained at

TABLE I

Characterization of Fatty Acids from Human Testes

Fatty acid	Retention time relative to methyl stearate	Product of hydrogenation	Longest λ absorption after alkaline isomerization, nm	Ozonolysis fragments[a]	
				Dicarboxylic	Monocarboxylic
20:3 ω6	3.0	Eicosanoic	n.d.[b]	C^{2}-8	C-6
22:4 ω6	6.0	Docosanoic	315	C^{2}-3, C^{2}-7	C-6
22:5 ω6	6.9	Docosanoic	346	C^{2}-3, C^{2}-4	C-6
22:5 ω3	8.0	Docosanoic	346	C^{2}-3, C^{2}-7	---
22:6 ω3	9.2	Docosanoic	375	C^{2}-3, C^{2}-4	---

[a] C^{2}-3 = propanedioic acid, C^{2}-4 = butanedioic acid, C^{2}-7 = heptanedioic acid, C^{2}-8 = octanedioic acid and C-6 = hexanoic acid.

[b] Not determined.

orchiectomy or autopsy. The tissue obtained at orchiectomy was fresh and was placed immediately in ice. Orchiectomy was performed as a prophylactic measure in cases of prostate cancer, and the testes were not involved. In the autopsy cases death was due to various causes, but testes were not involved. We have observed that concentrations of fatty acids of testes removed at orchiectomy are similar to those of testes removed at autopsy for the same age group (data to be published). In this report tissues from cases of ages 25-81 were included.

Fatty acid composition was determined for each case, and portions of each of seven pairs of testes showing typical fatty acid patterns were pooled for further analysis. The tissue was hydrolyzed under nitrogen in a mixture containing for each gram of tissue: 2 ml 40% KOH, 2 ml 95% ethanol and 0.2 ml 0.5% hydroquinone as antioxidant. The hydrolyzed mixture was chilled, acidified and extracted with petroleum ether. After washing the organic phase with 30% ethanol and drying with anhydrous sodium sulfate, the fatty acids were methylated, using BF_3, by the method of Metcalfe and Schmitz (5). Analysis of the methyl esters was done on a Varian 1520 gas chromatograph using a flame detector and a column packed with 15% EGSS-X on Gas Chrom P (Applied Science Lab., State College, Pa.).

Individual fatty acids (as methyl esters) were isolated by preparative gas chromatography in a Barber-Colman Model 10 gas chromatograph using a column packed with EGSS-X and equipped with a splitter, allowing collection of 80% of the effluent. Methyl esters, shown to be 95- 99% pure by analytical gas chromatography, were trapped in glass tubes containing silanized glass wool and were extracted from the glass wool with petroleum ether.

Individual methyl esters, isolated as described above, were hydrogenated by the method described by Farquhar et al. (6) and the resulting saturated methyl esters subjected to analytical gas chromatography as described previously. The number of double bonds in a purified fatty acid was determined by the maximum wavelength of UV absorption after alkaline isomerization, as described by Holman and Hayes (7). Purified methyl esters were subjected to oxidative ozonolysis by the method of Stoffel (8). The resulting fragments were methylated with BF_3-methanol reagent, as described previously, and analyzed by gas chromatography using a column temperature of 150 C.

RESULTS AND DISCUSSION

Fatty acid composition of testes obtained from patients suffering from prostate cancer did not differ substantially from that of testes obtained from autopsy specimens. Details of these analyses will be published separately. All tissue samples individually analyzed contained all five of the fatty acids isolated for characterization from the pooled tissue.

The results of the characterization of the five polyenes isolated are given in Table I. The following relative average amounts were obtained for these (in per cent of total fatty acids): 20:3ω6, 5.9; 22:4ω6, 1.3; 22:5ω6, 0.5; 22:5ω3, 0.6; and 22:6ω3, 8.0. The polyenes (20:4ω6 and 18:2ω6) were not characterized because of their known ubiquity. Propanedioic acid was observed in the chromatograms of the ozonized samples, although most of these products were lost in the ozonolysis procedure. The relative retention times of the native samples and of the hydrogenated derivative, the longest wave length absorption of the alkaline-isomerized acid and the fragments obtained on ozonolysis serve to firmly establish the structure of these five polyenoic acids.

Although the presence of 7,10,13,16,19-docosapentaenoic acid (22:5ω3) has been pre-

viously reported in beef and porcine testes (1), it has not been reported in human testes. The presence of this member of the α-linolenic acid family in human testes is not surprising in view of the relatively large amount of 22:6ω3 in human testis, the high probability of a precursor-product relationship between these fatty acids (9) and the substantial amounts of 22:5ω3 previously reported in ejaculated human sperm (10). The quite small relative amounts of 22:5ω3 and large relative amounts of 22:6ω3 present in human testes is in strong contrast to the large relative amounts of 22:5ω3 and small relative amounts of 22:6ω3 found by Ahluwalia and Holman (10) in human sperm. This may suggest extensive metabolism of these fatty acids during the latter stages of maturation and transport of sperm.

It is clear that, unlike the rat testis (2), human testis retains very little of its ω6 fatty acid as the 22-carbon polyenoic acids. One of the major polyenoic acids of rat testis is 22:5 ω6, and it has been shown that this fatty acid accumulates in rat testis during sexual maturation (2). Unlike the other species (1-3), human testis shows preference for the less abundant ω3 family when accumulating 22-carbon polyenes. It appears that these variations are not due to dietary differences but are characteristic of the species. It will be of interest to find the factors that are responsible for these differences.

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REFERENCES

1. Holman, R.T., and H.H. Hofstetter, JAOCS 42:540 (1965).
2. Davis, J.T., R.B. Bridges and J.G. Coniglio, Biochem. J. 98:342 (1966).
3. Bridges, R.B., and J.G. Coniglio, J. Biol. Chem. 245:46 (1970).
4. Bieri, J.G., and E.L. Prival, Comp. Biochem. Physiol. 15:275 (1965).
5. Metcalfe, L.D., and A.A. Schmitz, Anal. Chem. 33:363 (1961).
6. Farquhar, J.W., W. Insull, Jr., P. Rosen, W. Stoffel and E.H. Ahrens, Jr., Nutr. Rev. (Suppl.) 17(8): Part 2 (1959).
7. Holman, R.T., and H. Hayes, Methods Biochem. Anal. 4:99 (1957).
8. Stoffel, W., JAOCS 42:583 (1965).
9. Klenk, E., and H. Mohrhauer, Z. Physiol. Chem. 320:218 (1960).
10. Ahluwalia, B., and R.T. Holman, J. Reprod. Fert. 18:431 (1969).

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Influence of Vitamin C on Hydroxylation and Side Chain Oxidation of Cholesterol in Vitro

ABSTRACT

The addition of ascorbic acid (20-160 μg) to mitochondrial preparations of rat or guinea pig liver has no effect upon the oxidation of [26-^{14}C]-cholesterol to $^{14}CO_2$. The 7α-hydroxylation of cholesterol by rat liver microsomes is also unaffected by addition of ascorbic acid. Hydroxylation by guinea pig liver microsomes is increased in the presence of ascorbic acid, but the results are not statistically significant.

Ginter (1) has suggested that the rate of conversion of cholesterol to bile acids is correlated with the concentration of vitamin C in the liver. His conclusions are based on experiments that demonstrate that guinea pigs with latent vitamin C deficiency have significantly higher serum and liver cholesterol levels and convert less [26-^{14}C]-cholesterol to $^{14}CO_2$ than do control guinea pigs. The output of $^{14}CO_2$ is taken as the measure of bile acid formation.

If ascorbic acid has a stimulatory effect upon bile acid formation, it might be expected to exhibit this effect at the rate limiting step in the conversion of cholesterol to bile acids—7α-hydroxylation (2)—as well as at the level of side chain oxidation.

To test this hypothesis we have studied the 7α-hydroxylation of [1,2-^{3}H]-cholesterol by

viously reported in beef and porcine testes (1), it has not been reported in human testes. The presence of this member of the α-linolenic acid family in human testes is not surprising in view of the relatively large amount of 22:6ω3 in human testis, the high probability of a precursor-product relationship between these fatty acids (9) and the substantial amounts of 22:5ω3 previously reported in ejaculated human sperm (10). The quite small relative amounts of 22:5ω3 and large relative amounts of 22:6ω3 present in human testes is in strong contrast to the large relative amounts of 22:5ω3 and small relative amounts of 22:6ω3 found by Ahluwalia and Holman (10) in human sperm. This may suggest extensive metabolism of these fatty acids during the latter stages of maturation and transport of sperm.

It is clear that, unlike the rat testis (2), human testis retains very little of its ω6 fatty acid as the 22-carbon polyenoic acids. One of the major polyenoic acids of rat testis is 22:5 ω6, and it has been shown that this fatty acid accumulates in rat testis during sexual maturation (2). Unlike the other species (1-3), human testis shows preference for the less abundant ω3 family when accumulating 22-carbon polyenes. It appears that these variations are not due to dietary differences but are characteristic of the species. It will be of interest to find the factors that are responsible for these differences.

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REFERENCES

1. Holman, R.T., and H.H. Hofstetter, JAOCS 42:540 (1965).
2. Davis, J.T., R.B. Bridges and J.G. Coniglio, Biochem. J. 98:342 (1966).
3. Bridges, R.B., and J.G. Coniglio, J. Biol. Chem. 245:46 (1970).
4. Bieri, J.G., and E.L. Prival, Comp. Biochem. Physiol. 15:275 (1965).
5. Metcalfe, L.D., and A.A. Schmitz, Anal. Chem. 33:363 (1961).
6. Farquhar, J.W., W. Insull, Jr., P. Rosen, W. Stoffel and E.H. Ahrens, Jr., Nutr. Rev. (Suppl.) 17(8): Part 2 (1959).
7. Holman, R.T., and H. Hayes, Methods Biochem. Anal. 4:99 (1957).
8. Stoffel, W., JAOCS 42:583 (1965).
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If ascorbic acid has a stimulatory effect upon bile acid formation, it might be expected to exhibit this effect at the rate limiting step in the conversion of cholesterol to bile acids—7α-hydroxylation (2)—as well as at the level of side chain oxidation.

To test this hypothesis we have studied the 7α-hydroxylation of [1,2-^{3}H]-cholesterol by

TABLE I

Influence of Various Levels of Ascrobic Acid 7α-Hydroxylation of [1,2-^{3}H]-Cholesterol by Microsomes and Oxidation of [26-^{14}C]-Cholesterol to $^{14}CO_2$ by Mitochondria of Normal Guinea Pig and Rat Liver

Ascorbic acid, μg	7α-Hydroxylation, p mol/mg protein/min		[26-^{14}C]-Oxidation, p mol/mg N	
	Guinea pig[a]	Rat[b]	Guinea pig[a]	Rat[b]
0	1.10 ± 0.21	4.29 ± 1.25	4.1 ± 0.7	13.5 ± 2.5
20	1.21 ± 0.41	4.74 ± 1.04	4.0 ± 0.8	13.2 ± 2.5
40	1.61 ± 0.44	4.14 ± 0.96	3.7 ± 0.5	12.0 ± 1.8
80	1.81 ± 0.32	3.96 ± 0.83	3.8 ± 0.8	13.3 ± 2.7
160	1.94 ± 0.70	3.50 ± 1.03	3.7 ± 0.7	13.1 ± 2.2

[a]Average of four experiments ± SEM.
[b]Average of five experiments ± SEM.

microsomal preparations from rat and guinea pig liver and oxidation of [26-^{14}C]-cholesterol to $^{14}CO_2$ by suitably fortified mitochondrial preparations of rat and guinea pig liver.

Five rats and four guinea pigs were used. For preparation of microsomal enzyme, 6 g liver was homogenized in a solution of 0.25 M sucrose containing 0.075 M nicotinamide and 2.5 mM EDTA. Washed microsomes were prepared according to the method of Shefer et al. (3). Typical preparations of rat and guinea pig microsomes contained 12.1 and 11.6 mg/ml protein (4), respectively. [1,2-^{3}H]-Cholesterol (0.5 μmol; 8.6 x 10^5 cpm/μmol), solubilized in Tween 20, was incubated in a 25 ml Erlenmeyer flask containing potassium phosphate buffer, pH 7.4, 0.167 mmol; $MgCl_2$, 11 μmol; $NADP^+$, 3.0 μmol; glucose-6-phosphate, 6.0 μmol; glucose-6-phosphate dehydrogenase, 5 IU; and microsomal preparation, 1 ml. The final volume was 2.4 ml. Ascorbic acid was dissolved in buffer and the reaction mixtures contained 0.1 ml buffer containing 20, 40, 80 or 160 μg ascorbic acid; these levels of ascorbic acid correspond to 0.11, 0.23, 0.45 and 0.91 μmol, respectively. This range of concentrations was chosen because Ginter (1) found that control guinea pig liver contained 8.2 mg ascorbic acid per 100 g. The incubation was carried out by shaking at 37 C for 30 min. The reaction was stopped by the addition of 15 volumes methylene dichloride-ethanol 5:1, and the steroids extracted and separated by thin layer chromatography on Silica Gel G according to the method of Shefer et al. (5). The 7α-hydroxycholesterol band was scraped from the plate and assayed for radioactivity by liquid scintillation spectrometry.

Oxidation of [26-^{14}C]-cholesterol to $^{14}CO_2$ was carried out using previously published methods (6,7).

Incubations were carried out in stoppered 125 ml Erlenmeyer flasks containing center wells. The incubation mixture consisted of 1 ml mitochondrial preparation; 1 ml solution containing adenosine triphosphate (ATP, 25 mg), nicotinamide adenine dinucleotide (NAD, 5 mg), adenosine monophosphate (AMP, 8 mg), reduced glutathione (15 mg), sodium citrate monohydrate (30 mg), magnesium nitrate hexahydrate (10 mg), potassium penicillin G (2000 units) and streptomycin sulfate (1 mg); 5 ml labeled substrate ([26-^{14}C]-cholesterol, 0.8 nmol/incubation, 9.8 x 10^4 dpm/nmol) in 0.25 M tris (hydroxymethyl) aminomethane, HCl, pH 8.5; and 5 ml boiled supernatant.

Incubations were carried out at 37 C for 18 hr. At the end of this period 2.5 ml of a 1 M methanolic solution of Hyamine 10x (*p*-[diisobutyl-cresoxyethoxyethyl]-dimethybenzylammonium hydroxide) was injected into the center well. The solution was acidified with 1 N H_2SO_4 (2.5 ml) and the flasks were shaken for 3 hr at 37 C to displace $^{14}CO_2$. The Hyamine solution was removed from the center well and an aliquot taken for radioactive assay by liquid scintillation spectrometry.

Labeled substrates were purchased from the New England Nuclear Corporation (Boston, Mass.) and purified by thin layer chromatography prior to use.

The results obtained in this series of experiments are presented in Table I. It is evident that the addition of ascorbic acid did not affect in vitro oxidation of [26-^{14}C]-cholesterol by liver mitochondria from either guinea pigs or rats. We also compared the oxidation of [1-^{14}C]-octanoate (105 nmol per incubation; 7.4 x 10^3 cpm/nmol) to $^{14}CO_2$ by control mitochondrial preparations and by reaction mixtures containing 80 μg ascorbic acid. The thin layer chromatography was carried out on Silica Gel G using petroleum ether-ethyl ether-glacial acetic acid 83:16:1. The 7α-hydroxylation of cholesterol

by liver microsomes was unaffected by ascorbic acid in the rat. In the experiments with liver microsomes from the guinea pig, addition of ascorbic acid resulted in increased 7α-hydroxylation, but the differences were not significant.

Guchhait et al. (8) found that catabolism of cholesterol was decreased in scorbutic guinea pigs. They also reported that hepatic cholesterogenesis was enhanced in scorbutic guinea pigs and concluded that the increase in serum and liver cholesterol levels in vitamin C-deficient guinea pigs is due to a combination of increased cholesterol synthesis and decreased cholesterol catabolism. Guchhait et al. found that addition of ascorbic acid (1.0 mg) enhanced the conversion of [4-^{14}C]-cholesterol to bile acids by liver mitochondrial preparations from scorbutic or pair-fed normal guinea pigs. The stimulation of oxidation was much greater in the scorbutic animals (35 vs. 17% for pair-fed controls).

There is no question that cholesterol oxidation is impaired in guinea pigs with latent (1) or frank (8,9) vitamin C deficiency and that normal oxidation can be restored by resaturation with this vitamin. Although there is an apparent discrepancy between our in vitro results and the in vivo results of Ginter (1), it should be noted that our experiments were carried out under conditions strikingly different from Ginter's. The present data are not inconsistent with the hypothesis that there is a correlation between vitamin C concentration in liver and the rate of transformation of cholesterol to bile acids (1), but the correlation may hold only over a limited range of ascorbic acid concentrations. It would be of interest to explore this further with mitochondrial preparations from guinea pigs maintained at various suboptimal levels of vitamin C deficiency, but the present data suggest that supplementation of animals on an already adequate intake does not stimulate cholesterol oxidation.

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REFERENCES

1. Ginter, E., Science 179:702 (1973).
2. Bergström, S., and S. Lindstedt, Biochem. Biophys. Acta 19:556 (1956).
3. Shefer, S., S. Hauser and E.H. Mosbach, J. Lipid Res. 7:763 (1966).
4. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193:265 (1951).
5. Shefer, S., S. Hauser and E.H. Mosbach, J. Lipid Res. 9:328 (1968).
6. Whitehouse, M.W., E. Staple and S. Gurin, J. Biol. Chem. 234:276 (1959).
7. Kritchevsky, D., R.R. Kolman, M.W. Whitehouse, M.C. Cottrell and E. Staple, J. Lipid Res. 1:83 (1959).
8. Guchhait, R., B.C. Guha and N.C. Ganguli, Biochem. J. 86:193 (1963).
9. Ginter, E., R. Nemec and P. Bobek, Brit. J. Nutr. 28:205 (1972).

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Search for Prostaglandin A_1 in Onion

ABSTRACT

A fatty acid fraction separated from yellow onion has been subjected to thin layer chromatographic techniques, UV and IR spectrophotometric analyses. This silicic acid-chromatographed fraction corresponds to the prostaglandin A_1 fraction. Preliminary identification strongly suggests that this component of onions appears to be a prostaglandin (PGA_1) or a prostaglandin-like compound.

To date, there are no reports that prostaglandins have been isolated from plant materials. One attempt has been reported for a search of prostaglandins in plant material—wheat bran (1). However the extracted component was found to be a certain hydroxy-unsaturated acid, which behaved similarily to prostaglandins. In this investigation attempts have been made to characterize a fatty acid fraction that corresponds to prostaglandin A_1 (15-hydroxy-9-ketoprosta-10,13-dienoic acid or PGA_1).

Numerous plant materials were screened using thin layer chromatographic techniques to determine if such materials could possibly

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REFERENCES

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3. Shefer, S., S. Hauser and E.H. Mosbach, J. Lipid Res. 7:763 (1966).
4. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193:265 (1951).
5. Shefer, S., S. Hauser and E.H. Mosbach, J. Lipid Res. 9:328 (1968).
6. Whitehouse, M.W., E. Staple and S. Gurin, J. Biol. Chem. 234:276 (1959).
7. Kritchevsky, D., R.R. Kolman, M.W. Whitehouse, M.C. Cottrell and E. Staple, J. Lipid Res. 1:83 (1959).
8. Guchhait, R., B.C. Guha and N.C. Ganguli, Biochem. J. 86:193 (1963).
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Numerous plant materials were screened using thin layer chromatographic techniques to determine if such materials could possibly

TABLE I

R_f Values and Staining Results of Prostaglandins and Unknown Onion Component Corresponding to PGA_1 on Thin Layer Chromatographic Plates

Component	R_f[a]	R_f[b]	R_f[c]	UV light: Short	UV light: Long	PMA[d]	Iodine vapor[e]	CuOAc[f]
Onion extract	0.64	0.71	0.38	++	++	+	++++	+
PGA_1	0.64	0.72	0.38	+	+	+	+++	+
PGA_2	0.63	0.73	0.31	+	+	+	+++	+
PGE_1	0.49	0.48	0.21	–	–	+	+	+
$PGF_{2\alpha}$	0.32	0.27	0.10	–	–	+	+	+

[a]Silica Gel G thin layer chromatographic plates (chloroform-methanol-acetic acid 18:2:1 v/v).

[b]Silica Gel G thin layer chromatographic plates (ethyl acetate-ethanol-acetic acid 100:1:1 v/v).

[c]Silver nitrate-impregnated plates (chloroform-methanol-acetic acid 18:1:1).

[d]10% phosphomolybdic acid in 95% ethanol.

[e]This stain was not used on silver nitrate-impregnated plates.

[f]3% cupric acetate in 15% phosphoric acid.

contain prostaglandins. After this initial screening, onions were selected for further studies. From this screening it appeared that yellow onions (*Allium cepa*) might be a possible source for prostaglandins and the prostaglandin A_1 in particular. Also it is known that certain plant materials contain the necessary precursors of prostaglandin formation, such as linolenic acid and arachidonic acid (2). Finally, the consumption of onions or onion juice has been a home remedy for the treatment of high blood pressure, assuming that onions are capable of lowering blood pressure. The lowering of blood pressure is a well documented property of the prostaglandin A's (3).

The method used for the isolation of the lipid-soluble fatty acid fraction from onion was a procedure used by Lee et al. (4), modified for this investigation. In brief, the analysis used for the separation of the lipid-soluble fraction in onions was as follows. The sample was homogenized with buffer and the fibrous material removed by centrifugation; protein was removed by ethanol precipitation. After concentration the pH of the solution was adjusted and extractions were performed with petroleum ether. The prostaglandin fraction was finally extracted into chloroform. From a total of 3.4 kg onions, 0.8 g crude extract was obtained. This represented 10 separate analyses. This crude extract was then subjected to further tests and purification procedures: thin layer chromatography, biological assay and column chromatography.

The biological assay and the thin layer chromatographic procedures were used to determine if this crude fraction of onion contained a component that might correspond to a PGA. Observation of blood pressure profiles on pentobarbital-anaesthetized vagotomized pentolinium-treated rats with crude onion extract and PGA standards both indicated blood pressure lowering characteristics.

The thin layer chromatographic analyses are summarized in Table I. The results of these studies strongly indicate that a component in the crude onion extract corresponds to prostaglandin A_1.

The silicic acid column chromatographic purification procedure has been previously reported (5,6). The solvent system was benzene-ethyl acetate of the following compositions (v/v): 9:1, 4:1, 7:3, 2:3, 1:4 and 1:9. The benzene-ethyl acetate 4:1 fraction was taken as the one containing the prostaglandin A fraction. This was confirmed by using tritium-labeled PGA_1, which had been added to onions and subjected to the complete analysis and column chromatographic separation. Scintillation counting data clearly indicated that the 4:1 fraction contained PGA_1.

It is known that the PGA's have a maximum absorption at 217 nm. Also, when the PGA's are treated with base, the corresponding PGB is produced. The PGB's have a maximum absorption at 278 n. Base treatment and UV measurements were taken. The absorption profile at 278 nm in the benzene-ethyl acetate 4:1 fraction clearly indicated a component was present corresponding to a prostaglandin-like compound. Similar chromatograms are reported by Hamberg and Samuelsson (7) and Lee et al. (4).

TABLE II

IR Peak Assignments[a]

Peaks reported (8)	PGA_1 standard	Onion component	Structure
3.63	3.39	3.36	Solvent
3.71	3.47	3.41	CH_3, CH_2
3.78	3.56	3.45	OH
3.85	3.60	3.53	Solvent
5.86	5.84	5.82	C=O (ketonic)
5.88	5.89	5.88	C=O (ketonic)
6.30	6.30	6.30	C=C
8.13	7.94	7.82	CH_3
8.32	8.14	8.24	CH_3, COH
8.33	8.47	8.32	Solvent
9.80	9.80	9.63	COH, HC=CH (*trans*)
13.89	13.84	14.25	CH

[a]In microns.

The IR studies were performed on the purified PGA_1 fraction of the crude onion extract and were compared to PGA_1 standards. Table II summarizes the results of these studies. From the spectrum of the PGA_1 fraction of the onion extract, it appears that there is good evidence that this compound contains a monosubstituted α,β-unsaturated cyclopentanone ring.

Although the determination of the exact structure of the onion fatty acid fraction that corresponds to the PGA component is not conclusive for this study, the observations indicate that the probable structure of the fraction is very similar to PGA_1 or may be identical to it.

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REFERENCES

1. Albro, P.W., and L. Fishbein, Phytochemistry 10:631 (1971).
2. Mazur, A., and B. Harrow, "Textbook of Biochemistry," W.B. Saunders Co., Philadelphia, Pa., 1971, p. 309.
3. Lee, J.B., J.C. McGiff, H. Kannegiesser, Y.Y. Aykent, J.G. Mudd and T.F. Frawley, Ann. Int. Med. 74:703 (1971).
4. Lee, J.B., K. Crowshaw, B.H. Takman, K.A. Attrep and J.Z. Gougoutas, Biochem. J. 105:1251 (1967).
5. Hirsch, J., and E.H. Ahrens, J. Biol. Chem. 233:311 (1958).
6. Samuelsson, B., Ibid. 238:3229 (1963).
7. Hamberg, M., and B. Samuelsson, Ibid. 241:257 (1966).
8. Pike, J.E., F.H. Lincoln and W.P. Schneider, J. Org. Chem. 34:3552 (1969).

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Paraffinic Hydrocarbon Composition of Earthworms (*Lumbricus terrestris*)

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ABSTRACT

The paraffinic hydrocarbon fraction of the lipids extracted from earthworms (*Lumbricus terrestris*) contains ca. 88% *n*-alkanes and 12% branched alkanes and other hydrocarbons. The *n*-alkanes range from C_{13} through C_{33}, and their most interesting feature is the even-over-odd carbon number predominance in the C_{13}-C_{24} range. The nonnormal hydrocarbons consist primarily of monomethyl-substituted alkanes. Other hydrocarbons that have been identified include the C_{16}, C_{18}, C_{19} and C_{20} isoprenoids and the C_{18}, C_{20} and C_{22} *n*-alkylcyclohexanes.

INTRODUCTION

The previous research (1) showed that the earthworms (*Lumbricus terrestris*) contained 0.65-3.5% lipids (based on fresh weight). The average content of lipids was 1.0-1.5%. Saponification of the crude lipid extract yielded 32% fatty acids, 25% unsaponifiables and 43% unidentified. The lipid contained 3% hydrocarbon and 16% sterols. Cursory gas liquid chromatography (GLC) of the hydrocarbons showed at least 13 components.

This paper describes the analyses of the earthworm hydrocarbon fraction using modern

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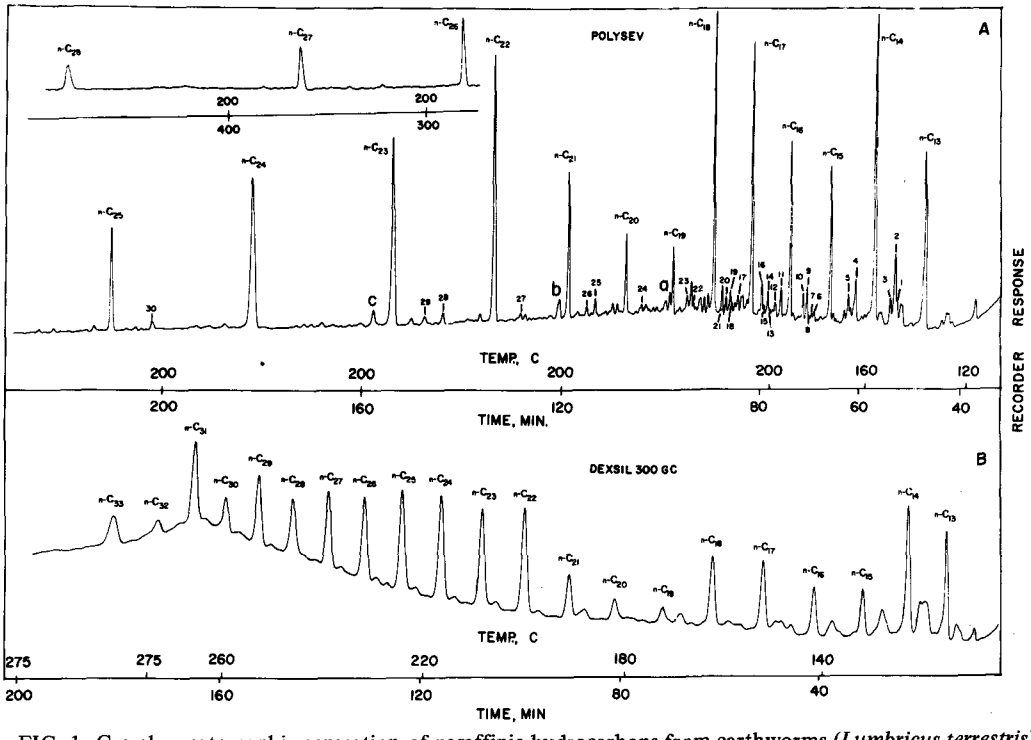

FIG. 1. Gas chromatographic separation of paraffinic hydrocarbons from earthworms (*Lumbricus terrestris*). A. Stainless steel tubing, 152.4 m x 0.051 cm ID, coated with Polysev; Varian 1200 gas chromatograph equipped with flame ionization detector; helium flow, 5 ml/min; no split; isothermal at 60 C for 10 min, then programed at ca. 2 C/min to 200 C; ca. 6 x 10^{-6} g aliphatic hydrocarbons injected; range, 10; attenuation, 1. B. Glass tubing, 1.8 m x 0.4 cm ID, packed with 5% Dexsil 300 GC on Chromosorb G; Varian 1200 gas chromatograph equipped with a flame ionization detector; helium flow, 8 ml/min; no split; programed from 100 to 275 C at ca. 1 C min; ca. 24 x 10^{-6} g of aliphatic hydrocarbons injected; range, 10; attenuation, 8.

TABLE I

Normal Alkanes Identified by Gas Chromatography-Mass Spectrometry

Carbon no.	Relative %
C_{13}	2.9
C_{14}	5.0
C_{15}	2.3
C_{16}	2.7
C_{17}	3.2
C_{18}	3.6
C_{19}	0.9
C_{20}	1.4
C_{21}	2.5
C_{22}	6.9
C_{23}	6.2
C_{24}	6.7
C_{25}	7.1
C_{26}	5.7
C_{27}	6.2
C_{28}	5.3
C_{29}	5.9
C_{30}	2.4
C_{31}	6.7
C_{32}	1.1
C_{33}	3.6

methods.

EXPERIMENTAL PROCEDURES

Paraffinic Hydrocarbon Fraction

The earthworms (*Lumbricus terrestris*) were collected at Boyertown, Pa. Lipid extractions from earthworms and further separation of lipids were carried out as described in the previous paper (1). The hydrocarbons were obtained by fractionation of unsaponifiable material from the total lipid extract. The chromatographic procedures used (1) insured that the hydrocarbon fraction recovered contained essentially only paraffinic hydrocarbons.

Gas Chromatography and Gas Chromatography-Mass Spectrometry

Aliquots of the hydrocarbon fraction were dissolved in benzene or hexane. One to four microliters of these solutions were used in subsequent analyses.

Gas chromatographic analyses were carried out on a Varian 1200 FID gas chromatograph fitted with either a stainless steel capillary (152.4 m x 0.051 cm ID) coated with Polysev (*m*-bis-*m*-[phenoxyphenoxy]-phenoxybenzene obtained from Applied Science Lab., Inc., State College, Pa. 16801) or a glass column (1.8 m x 0.4 cm ID) packed with 5% Dexsil 300 GC (polycarborane siloxane obtained from Analabs, Inc., New Haven, Conn. 96473) on Chromosorb G. Tentative identification of the normal hydrocarbons was made by comparing the chromatograms of the sample with chromatograms of a known mixture of hydrocarbons obtained under identical conditions on the same instrument.

Gas chromatographic-mass spectrometric (GC-MS) analyses were made using an LKB 9000 gas chromatograph-mass spectrometer combination fitted with a stainless steel capillary (152.4 m x 0.051 cm ID) coated with Polysev or a stainless steel column (1.5 m x 0.2 cm ID packed with 3% SE-30 on Varaport 30) obtained from Varian Associates, Pala Alto, Calif. 94303. Compound identifications were assigned from spectra taken when separated components of the hydrocarbon mixture were eluted into the mass spectrometer.

The relative amounts of individual hydrocarbons extracted from earthworms are expressed as percentages of the weights of hydrocarbons estimated from the areas under the peaks of the chromatograms. The weights were obtained as follows:

$$W_i = A_i \frac{w_j}{a_j}$$

where: W_i = weight of the ith alkane in the injected sample; A_i = area of the ith peak of the chromatogram of the sample; w_j = weight of the jth alkane in the injected standard; and a_j = area of the jth peak of the chromatogram of the standard.

RESULTS

The profiles of aliphatic hydrocarbons extracted from earthworms are shown in Figure 1. The chromatographic pattern obtained using an open tubular column coated with Polysev is presented as Figure 1A, the pattern obtained with a short packed column employing the high temperature phase Dexsil 300 GC as Figure 1B.

Normal Alkanes

The lipids from earthworms contain 3% hydrocarbons (1). About 88% of these hydrocarbons are normal alkanes (Table I). The normal alkanes range from C_{13} through C_{33}. A distinctive feature of the distribution is the predominance of even-numbered-carbon alkanes in the C_{13}-C_{24} range. In this range the carbon preference index (CPI) (2) is 0.7. Normal alkanes occurring in nature most often have a CPI $\simeq$ 1, e.g., in petroleum (3), or $>$ 1,

TABLE II

Methyl-Branched Alkanes Identified by Gas Chromatography-Mass Spectrometry

Peak designation in Figure 1A	Hydrocarbon	Carbon no.	Relative %
1	4-Methyltridecane	C_{14}	0.3
2	2-Methyltridecane	C_{14}	1.1
3	3-Methyltridecane	C_{14}	0.3
4	2,6,10-Trimethyltridecane	C_{16}	1.0
5	2-Methyltetradecane	C_{15}	0.5
6	6- and 7-Methylpentadecanes	C_{16}	0.2
7	5-Methylpentadecane	C_{16}	0.1
8	4-Methylpentadecane	C_{16}	0.1
9	2-Methylpentadecane	C_{16}	0.5
10	3-Methylpentadecane	C_{16}	0.4
11	2,6,10-Trimethylpentadecane	C_{18}	0.5
12	6-, 7- and 8-Methylhexadecanes	C_{17}	0.3
13	4-Methylhexadecane	C_{17}	0.1
14	2-Methylhexadecane	C_{17}	0.5
15	3-Methylhexadecane	C_{17}	0.3
16	2,6,10,14-Tetramethylpentadecane	C_{19}	0.7
17	6-, 7-, 8- and 9-Methylheptadecanes	C_{18}	0.2
18	4-Methylheptadecane	C_{18}	0.1
19	2-Methylheptadecane	C_{18}	0.1
20	3-Methylheptadecane	C_{18}	0.2
21	2,6,10,14-Tetramethylhexadecane	C_{20}	0.5
22	4-Methyloctadecane	C_{19}	0.1
23	3-Methyloctadecane	C_{19}	0.2
24	3-Methylnonadecane	C_{20}	0.1
25	2-Methyleicosane	C_{21}	0.3
26	3-Methyleicosane	C_{21}	0.2
27	3-Methylheneicosane	C_{22}	0.1
28	2-Methyldocosane	C_{23}	0.2
29	3-Methyldocosane	C_{23}	0.1
30	2-Methyltetracosane	C_{25}	0.2

e.g., in higher plants (4). In the C_{25}-C_{33} range the odd-numbered-carbon alkanes are present in larger amounts, giving a CPI of 1.9.

Branched Alkanes

About 11% of the paraffinic hydrocarbons are methyl-branched alkanes. The methyl-branched alkanes that have been identified by GC-MS are listed in Table II. In addition to the monomethyl-substituted alkanes, which are those typically found in Fischer-Tropsch synthesis (5,6; Nooner, D.W., J. Gilbert, E. Gelpi and G. Oró, in preparation), the earthworm hydrocarbons include the C_{16}, C_{18}, C_{19} and C_{20} isoprenoids (peaks 4, 11, 16 and 21, Fig. 1A) and the C_{18}, C_{20} and C_{22} monocyclohexylalkanes (peaks a, b and c, Fig. 1A).

Mass spectra show that the peaks labeled *n*-C_{20}, *n*-C_{30} and *n*-C_{32} in Figure 1 represent, in addition to the normal hydrocarbons, substantial amounts of branched aliphatic hydrocarbons. The hydrocarbon associated with *n*-eicosane appears to be a highly branched alkane and those associated with *n*-triacontane and *n*-dotriacontane to be 2-methylnonacosane and 2-methylhentriacontane, respectively.

By holding the maximum temperature of the Polysev-coated capillary at 150 C, the branched alkane associated with *n*-eicosane was resolved as a small peak immediately preceding the *n*-eicosane. The ratio of normal to branched alkane was ca. 5:1. The mass spectrum of the separated hydrocarbon did not permit its unequivocal identification. Major fragment ions were observed at m/e = 127 $[C_9H_{19}]^+$, 239 $[C_{17}H_{35}]^+$ and 267 $[C_{19}H_{39}]^+$. However no molecular ion was detected.

DISCUSSION

As previously suggested (1), the primary source of the hydrocarbons found in earthworms is probably soil humus. The leaves of plants that contribute much to soil humus, e.g., pasture plants (7), grasses (8), hardwoods (8), contain relatively large amounts of higher molecular weight hydrocarbons (C_{25}-C_{33}) in their surface waxes. That these hydrocarbons can survive and accumulate in the humus is attested

by their occurrence in soil (8), peat (8) and soft brown coals (9) with distributions not too dissimilar from those observed in the aforementioned plants.

The lower molecular weight hydrocarbons (C_{13}-C_{24}) found in earthworms also probably originated in the soil humus. However their origin cannot be explained as readily as that of the higher molecular weight hydrocarbons, since the even-over-odd carbon number preference observed is the exception rather than the rule in nature. Profiles in which even-numbered-carbon alkanes predominate in medium or low range distribution have been observed in sheep's wool (10) and Recent sediments from the Persian Gulf (11). In these cases, bacterial modification via oxidation and decarboxylation of existing alkanes (10) or reduction of even-numbered-carbon fatty acids (11) were suggested as possible reasons for the even-numbered-carbon hydrocarbon predominance.

Both of these mechanisms may be operative in forming the even-over-odd carbon number features of the *n*-alkane distribution observed in earthworms. There is a slight correlation between the normal alkanes and saturated normal fatty acids (1) in the C_{13}-C_{24} range, but it is of little help in selecting a mechanism of hydrocarbon formation. We believe the specific hydrocarbon distribution observed in the C_{13}-C_{24} range is the result of microbial activity, regardless of the mechanism that produces individual hydrocarbons.

The preceding are attempts to explain the hydrocarbons found in earthworms. Definitive answers to questions regarding the hydrocarbons found in earthworms will require additional work. A comparative study of cattle (*Bos taurus*) feed (7) and feces (12) showed that hydrocarbons ingested passed through the animal unchanged. A similar, more extensive study could be carried out with earthworms and would consist of comparative analyses of the hydrocarbons in earthworms, their food, environment and castings. Another approach would be to study the hydrocarbons in earthworms grown in the laboratory under controlled conditions.

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REFERENCES

1. Cerbulis, J., and M.W. Taylor, Lipids 4:363 (1969).
2. Cooper, J.E., and E.E. Bray, Geochim. Cosmochim. Acta 27:1113 (1963).
3. Bray, E.E., and E.D. Evans, Ibid. 22:2 (1961).
4. Eglinton, G., and R.J. Hamilton in "Chemical Plant Taxonomy," Edited by T. Swain, Academic Press, London, 1963, p. 187.
5. Studier, M.H., R. Hayatsu and E. Anders, Geochim. Cosmochim. Acta 32:151 (1968).
6. Gelpi, E., J. Han, D.W. Nooner and J. Oró, Ibid. 34:965 (1970).
7. Oró, J., D.W. Nooner and S.A. Wikström, Science 147:870 (1965).
8. Nooner, D.W., Ph.D. dissertation, University of Houston, Houston, Tex., 1966, p. 174.
9. Brooks, J.D., and J.W. Smith, Geochim. Cosmochim. Acta 31:2389 (1967).
10. Simmonds, P.G., D.W. Nooner, A. Zlatkis and J. Oró, JAOCS 45:34 (1968).
11. Welte, D.H. and G. Ebhardt, Geochim. Cosmochim. Acta 32:465 (1968).
12. Oró, J., D.W. Nooner and S.A. Wikström, J. Gas Chromatogr. 3:105 (1965).

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Variation with Chain Length in Acute Toxicity of Alkylhydroxamic Acids to Salmon (*Salmo salar*) Fry[1]

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ABSTRACT

The acute toxicity of saturated *n*-alkylhydroxamic acids to salmon (*Salmo salar*) fry was determined. Within the series *n*-$C_6H_{13}CONHOH$ to *n*-$C_{10}H_{21}CONHOH$, acute toxicity (indicated by death) increased with chain length. Shorter and longer chain compounds were not toxic under similar conditions. Factors governing the variation in toxicity with chain length and possible mechanisms of action are discussed.

INTRODUCTION

Fatty acids and some of their derivatives such as fatty amines have had applications as ore flotation agents (1). Recently a number of reports have appeared in the Russian literature describing the use of hydroxamic acids as flotation agents (2-6). These compounds, of structure RCONHOH where R is an alkyl group, are prepared by condensation of the appropriate carboxylic acid methyl ester with hydroxylamine. The flotation agent of this type most usually described (IM-50) is a mixture of sodium salts of medium chain length hydroxamic acids.

In an earlier paper (7), we described the acute toxicity to brook trout of a similar flotation agent used experimentally in Canada. This material consisted of a mixture of saturated hydroxamic acids of medium chain length (principally $C_7H_{15}CONHOH$ and $C_9H_{19}CONHOH$ but with traces of shorter and longer chain homologs) prepared from a low boiling fraction of coconut oil methyl esters, partially neutralized with dimethylamine and suspended in isopropanol. Toxicity was attributed to the hydroxamic acid (or hydroxamate anion) rather than to dimethylamine or to hydroxylamine (a potential decomposition product). In the course of this work we observed that *n*-decanohydroxamic acid, tested as its sodium salt, was considerably more toxic than the flotation agent itself. The possibility that the toxicity of the hydroxamic acids might vary with chain length was investigated as described below.

EXPERIMENTAL PROCEDURES

n-Alkylhydroxamic acids ranging in chain length from $CH_3CONHOH$ to $C_{15}H_{31}CONHOH$ were synthesized. Potassium salts of $C_9H_{19}CONHOH$ and higher acids were prepared as described by Blatt (8) for benzohydroxamic acid, starting with the appropriate methyl ester (purchased from Eastman Kodak or K and K Labs., Inc., or prepared from the acid [Aldrich] with boron trifluoride-methanol). The potassium salts were dissolved or suspended in water, acidified, and the free acid was extracted into diethyl ether. Hydroxamic acids of chain length lower than $C_9H_{19}CONHOH$ were prepared by the method of Fishbein et al. (9). All hydroxamic acids were recrystallized from ethyl acetate to constant melting point.

The acute toxicity of these compounds to salmon (*Salmo salar*) fry was determined. Fish from 5-7 cm in length were taken from the laboratory's stock maintained in fresh water at 10 C. They were placed at random in groups of five in fiberglass tanks containing 10 liters continuously aerated fresh water at 10 C and allowed to adjust to these conditions for some hours before exposure to toxicant. The hydroxamic acids tested were added to the tanks as solutions or suspensions in 10 ml isopropanol (Fisher, reagent grade) to give final concentrations in the range 1-100 ppm; an isopropanol control was run in all such tests. Tests were also carried out on hydroxylamine hydrochloride (Baker, reagent grade) and on sodium nitrite (Fisher, reagent grade), since hydroxylamine and nitrite were remotely possible decomposition products of hydroxamic acids (discussed further below). Both compounds were administered as solutions in 10 ml water; the hydroxylamine hydrochloride solution was brought to pH 7 by addition of 10N NaOH. Fish were inspected at intervals of 15 min during the first few hours of exposure, and thereafter at gradually increasing intervals. They were not fed while under test. Dead fish were removed, and the time to death recorded. LT50's (time to

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

TABLE I

Physical Data for *n*-Alkylhydroxamic Acids

Acid	Melting point, C		K_{water/CCl_4}, 25 C
	Observed	Literature	
$CH_3CONHOH$	90	89-91[a]	ND[b]
$C_3H_7CONHOH$	38	---	ND[b]
$C_5H_{11}CONHOH$	64	64[c]	430
$C_6H_{13}CONHOH$	73	71.5-72[d]	245
$C_7H_{15}CONHOH$	79	79[c]	16
$C_8H_{17}CONHOH$	83	81.5-82[d]	3.5
$C_9H_{19}CONHOH$	88	88[c]	0.98
$C_{10}H_{21}CONHOH$	92	90[c]	0.21
$C_{11}H_{23}CONHOH$	94	93[c]	0.05
$C_{12}H_{25}CONHOH$	97	---	0.02
$C_{13}H_{27}CONHOH$	99	99[e]	ND
$C_{15}H_{31}CONHOH$	104	99[c]	ND

[a](9).
[b]Not determined.
[c](12).
[d](16).
[e](21).

death for 50% of the test group) were calculated by probit analysis (10).

Dissolved oxygen, pH and hydroxamic acid concentration were monitored at 24 hr intervals, starting 30 min after the addition of toxicant. For hydroxamic acid determinations, samples were filtered through Whatman No. 42 paper, and the filtrates analyzed by the method of Nery (11) or, where concentrations were sufficiently high, by measurement of the absorbance of the Fe^{+++} complex (9). Standard solutions of hydroxamic acids were made up in water, or in the case of acids higher than $C_9H_{19}CONHOH$, in 25% isopropanol in water. Use of isopropanol facilitated solution of the longer chain compounds and did not interfere with color development in either method or with the oxidation step in Nery's method (11), as judged from tests on shorter chain acids. In later stages of the work, hydroxylamine and nitrite were determined simultaneously with hydroxamic acids, using Nery's method (11) with oxidation at pH 3.5 and without oxidation, respectively. All determinations were made on a Unicam SP-500 instrument fitted with 1 cm cells.

Distribution coefficients of several of the hydroxamic acids between distilled water and carbon tetrachloride (Fisher, reagent grade) at 25 C were estimated by shaking solutions of the acid in either solvent with equal volumes of the other phase at intervals over 24 hr. Aliquots of the water phase, and of the carbon tetrachloride phase taken to dryness under N_2 and redissolved in water or aqueous isopropanol, were analyzed by Nery's method (11).

RESULTS

Melting points and other analytical data for the hydroxamic acids synthesized are shown in Table I, together with comparable data from the literature. Most observed melting points were in good agreement with literature values, but reference data were not available for $C_3H_7CONHOH$ and $C_{12}H_{25}CONHOH$, and the observed melting point of $C_{15}H_{31}CONHOH$ differed considerably from the literature value (12). Elemental analyses performed on these compounds yielded results in good agreement with theoretical values. $C_4H_9O_2N$–Calculated: %C, 46.60; %H, 8.74; %O, 31.07; %N, 13.59. Found: %C, 47.18; %H, 8.72; %O, 30.70; %N, 13.22. $C_{13}H_{27}O_2N$–Calculated: %C, 68.12; %H, 11.79; %O, 13.97; %N, 6.11. Found: %C, 68.17; %H, 11.78; %O, 13.34; %N, 6.08. $C_{16}H_{33}O_2N$–%C, 70.85; %H, 12.18; %O, 11.81; %N, 5.17. Found: %C, 71.51; %H, 12.27; %O, 11.89; %N, 5.21.

Only those acids in the series $C_6H_{13}CONHOH$ to $C_{10}H_{21}CONHOH$ were lethal at concentrations of 100 ppm or less within 96 hr. Graphs of LT50 vs. concentration for each acid in the series are shown in Figure 1, and parameters computed for a power function relationship ($y = ax^b$) between these variables are summarized in Table II. (All data in Table II and Fig. 1 refer to measured concentrations in

TABLE II

Parameters Estimated for Regression of LT50 on Concentration for Hydroxylamine and Alkylhydroxamic Acids[a]

Compound	a	b (±SE)	r
Hydroxylamine (free base)	94	-1.23 ± 0.17[b]	−0.99
$C_6H_{13}CONHOH$	31303	−1.61	(−1.0)[d]
$C_7H_{15}CONHOH$	222	-0.86 ± 0.35[b,c]	−0.93
$C_8H_{17}CONHOH$	41	-0.78 ± 0.56[b,c]	−0.81
$C_9H_{19}CONHOH$	15	-0.75 ± 0.15[c]	−0.99
$C_{10}H_{21}CONHOH$	4.8	-0.31 ± 0.21[c]	−0.88

[a]Equation is of form $y = ax^b$, where y = LT50 (hr), x = concentration (ppm), and a and b are fitted coefficients.

[b,c]Slopes having same superscript are not significantly different ($P<0.05$).

[d]Two data points only.

solution, rather than to concentrations added.) Within the series, toxicity increased with chain length, as illustrated by the decrease in 24 hr. LC50s (concentration in ppm required to produce a 50% kill in 24 hr, calculated from the data in Table II) with increasing chain length were as follows: $C_6H_{13}CONHOH$, 86; $C_7H_{15}CONHOH$, 13.3; $C_8H_{17}CONHOH$, 1.99; $C_9H_{19}CONHOH$, 0.53; $C_{10}H_{21}CONHOH$, 0.006.

Acids lower than $C_6H_{13}CONHOH$ were not lethal at concentrations below 100 ppm within 96 hr. Acids higher than $C_{10}H_{21}CONHOH$ added at concentrations of ca. 10 ppm or above were also not lethal within this period, but measured concentrations of these materials in solution ranged from not detectable (<0.2 ppm) to ca. 0.5 ppm. Fish exposed to the toxic acids all died in the same manner. Following a brief period of apparent excitability, they seemed to lose balance, gill movements slowed, and they died with the opercula open as if death were attributable to asphyxiation.

The toxicity of hydroxylamine (calculated as free base) was less than that of the more toxic acids, but still appreciable (Table II, Fig. 1). Sodium nitrite was of relatively low toxicity, producing an LT50 of 20 hr at 114 ppm (calculated as nitrite ion). No control fish (exposed to isopropanol at 1000 ppm) died.

Throughout the tests, dissolved oxygen levels were close to saturation, and pH was within the range 6.8-7.2. Similar variation in pH was observed in the controls. Hydroxamic acid concentrations did not vary appreciably during the first 48 hr of test but by 96 hr were generally ca. 15-20% below starting concentrations. The discrepancy could not be attributed to decomposition to hydroxylamine or to nitrite, as these components did not increase above control or blank values. Analysis of control solutions showed that hydroxamic acid and hydroxylamine were not present at detectable concentrations and that nitrite was present at concentrations below 1 ppm.

Distribution coefficient measurements (Table I) showed that the acid $C_9H_{19}CONHOH$ was approximately equally distributed between water and carbon tetrachloride, and that shorter and longer homologs tended to enter the aqueous and organic phases, respectively. Estimates of very high and very low partition coefficients involved measurements of low hydroxamate concentrations and are necessarily less precise than intermediate values.

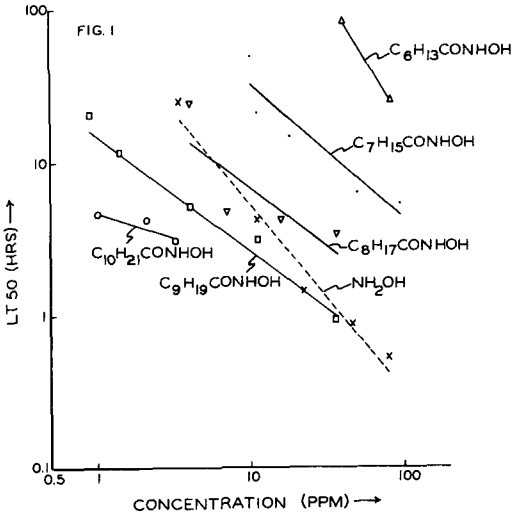

FIG. 1. Graphs of LT50 (hr) vs. concentrations (ppm) for toxicity of hydroxamic acids n-C_6H_{13}CONHOH to n-$C_{10}H_{21}$CONHOH towards salmon (*Salmo salar*) fry. Equations describing these curves are summarized in Table II. △, $C_6H_{13}CONHOH$; •, $C_7H_{15}CONHOH$; ▽, $C_8H_{17}CONHOH$; □, $C_9H_{19}CONHOH$; ○, $C_{10}H_{21}CONHOH$; X, NH_2OH.

DISCUSSION

The increase in acute toxicity of alkylhydroxamic acids with chain length explains our previous observation that the acid C_9H_{19} CONHOH was more toxic than a flotation agent based on a mixture of ca. 60% C_7H_{15} CONHOH and 30% C_9H_{19}CONHOH; the difference can be ascribed to the presence in the mixture of a large proportion of the less toxic acid. The data also confirm our previous suggestion that the toxic component in the flotation agent was hydroxamic acid (or hydroxamate ion) rather than the possible (although improbable) decomposition products, hydroxylamine or nitrite. No hydroxylamine or nitrite was detected in test solutions of hydroxamic acids, in agreement with the expectation that these acids would not decompose except at extremes of pH (13). In any case, hydroxylamine was not sufficiently toxic to account for the toxicity through decomposition of the longer chain compounds (C_8H_{17}CONHOH to $C_{10}H_{21}$ CONHOH); on a molar basis, it was slightly more toxic than C_7H_{15}CONHOH but not as toxic as C_8H_{17}CONHOH or higher acids. Nitrite was not sufficiently toxic to account for the toxicity of any of the hydroxamic acids.

The absence of acute toxicity within 96 hr from hydroxamic acids higher than $C_{10}H_{21}$ CONHOH was surprising. Regression of 24 hr LC50's (summarized above) on chain length, and projected to $C_{11}H_{23}$CONHOH, $C_{12}H_{25}$CONHOH, $C_{13}H_{27}$CONHOH and $C_{15}H_{31}$CONHOH, showed that these acids should have had 24 hr LC50's of 0.04, 0.01, 0.004 and 0.0005 ppm, respectively. These concentrations are well below the minimum detectable limit (ca. 0.2 ppm), but possibly not below the limit of solubility of these compounds. Analysis of test solutions to which 10 ppm $C_{13}H_{27}$CONHOH or $C_{15}H_{31}$CONHOH had been added did not show any hydroxamate in solution. However analysis of corresponding solutions of the $C_{11}H_{23}$CONHOH and $C_{12}H_{25}$CONHOH solutions showed apparent concentrations of these acids at ca. 0.5 ppm. We can only conclude that the latter data reflect analytical imprecision at this level, and that in general the absence of acute toxicity from the longer chain acids reflects their insolubility and failure to reach high enough concentrations in solution to be effective.

The slope of the dose-response curve for hydroxylamine was significantly greater than those of the higher hydroxamic acids ($p<0.05$; Table II), but slopes of the curves for the hydroxamic acids did not differ significantly from one another. Although this reflects the high variance of the data presented here, it is worth noting that similarity in the slopes of such curves for various compounds is often taken as representing similar mechanisms of action (14). In view of their structural similarity, it would be reasonable to conclude that all the hydroxamic acids tested did indeed act in the same way.

An increase in toxicity with chain length is not unique to the alkylhydroxamic acids. Similar variations in toxicity (and in other forms of biological activity) with chain length have been observed in the effect of several series of compounds on various organisms (15). The phenomenon can be explained by the increased lipid solubility of the longer chain homologs, enhancing their partition into a relatively lipoid biophase from the surrounding medium. For the hydroxamic acids, the relationship between toxicity and lipid solubility was expressed quantitatively by plotting the 24 hr *LC50* (see Results) against partition coefficient (Table I, final column). A linear equation of the form $y = 0.3.44x + 2.09$ (y = 24 hr LC50, ppm; $x = K_{water/CCl_4}$) fitted the data best ($r = 0.996$). It is realized, of course, that to envisage a fish in water as a simple biphasic system is a considerable simplification. It should also be emphasized that the relationship outlined above merely described the data, but does not necessarily define the processes underlying the toxic action of the hydroxamic acids. It suggests, however, that the toxicity of the hydroxamic acids depends on their being absorbed by the fish. The evidence for this is very limited; it is based solely on the observation that hydroxamic acid concentration in test solutions fell slightly over a 96 hr period. Further work is under way to establish whether or not absorption of these compounds does indeed occur.

Assuming that the hydroxamic acids are absorbed by the fish, it is of interest to consider the physiological basis of their toxicity. The literature describes a number of demonstrations of biological activity by these compounds; the best documented of these is that of urease inhibition. Most short and medium chain acids are powerful urease inhibitors (16-18). However, as this enzyme is probably absent from teleosts (as it is from most animals [19] except as a component enzyme of the microorganisms of the gut flora [18]), it is difficult to explain hydroxamic acid toxicity in terms of urease inhibition. Hydroxamic acids also have antifungal and antibacterial activity (12), but the mechanisms underlying this form of activity have not been explained; however they appear not to be based on urease inhibition. The only other form of biological activity that appears to

have been reported is carcinogenicity (in the homolog $C_{17}H_{35}CONHOH$ [20]) but the short LT50's observed in some tests can hardly be ascribed to such a process. Indeed, consideration of the symptoms observed at death and the very short LT50's caused by some compounds suggests that the mode of action of these compounds involves interference with some fundamental process such as respiration or nervous control. The mechanism remains to be established.

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REFERENCES

1. Frommer, D.W., JAOCS 47:189 (1970).
2. Gorlorskii, S.I., N.S. Khobotova and N.E. Shchukina, Chem. Abstr. 65:363b (1966).
3. Gorlorskii, S.I., N.P. Khobotova and N.E. Shchukina, Ibid. 66:4983g (1967).
4. Gorlorskii, S.I., N.P. Khobotova and N.E. Shchukina, Ibid. 69:98604x, (1968).
5. Strel'tsin, V.G., and N.P. Khobotova, Ibid. 70:117275e (1969).
6. Koltunova, T.E., and O.S. Bogdanov, Ibid. 76:363304 (1972).
7. Fletcher, G.L., and R.F. Addison, Bull. Env. Contam. Toxicol. 7:147 (1972).
8. Blatt, A.H., "Organic Syntheses," Collective vol. 2, Wiley & Sons Inc., New York, 1943, p. 67.
9. Fishbein, W.N., J. Daly and C.L. Streeter, Anal. Biochem. 28:13 (1969).
10. Finney, D.J., "Probit Analysis," Second edition, Cambridge University Press, 1952.
11. Nery, R., Analyst 91:388 (1966).
12. Hase, J., K. Kobashi, N. Kawaguchi and K. Sakamoto, Chem. Pharm. Bull. 19:363 (1971).
13. Mathis, F., Bull. Soc. Chim. Fr. 1953:D9.
14. Sprague, J.B., Water Res. 3:793 (1969).
15. O'Brien, R.D., "Insecticides, Action and Metabolism," Academic Press, New York, 1967, Chapter 2.
16. Hase, J., and K. Kobashi, J. Biochem. 62:293 (1967).
17. Kobashi, K., K. Kumaki and J. Hase, Biochim. Biophys. Acta 227:429 (1971).
18. Fishbein, W.N., P.P. Carbone and H.D. Hochstein, Nature 208:46 (1965).
19. Goldstein, L., and R.P. Forster, in "Comparative Biochemistry of Nitrogen Metabolism," Vol. 2, Edited by J.W. Campbell, Academic Press, New York, 1970, Chapter 10.
20. Swern, D., R. Weider, M. McDonough, D.R. Meranze and M.B. Shimkin, Cancer Res. 30:1037 (1970).
21. Babu, B.H., S.V. Rao and K.T. Achaya, Indian J. Technol. 5:327 (1967).

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Studies on in Vitro Peroxidation of Liver Lipids in Ethanol-Treated Rats

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ABSTRACT

It has been confirmed that lipid peroxidation of liver homogenates is increased following acute ethanol intoxication. Studies with recombined fractions of the liver suggest that the cause for the ethanol-induced increase in malonic dialdehyde production is located in the soluble fraction. Reduced glutathione content of the liver supernatant is decreased following ethanol intoxication. The decrement, however, occurs after the increase in malonic dialdehyde production takes place. Glutathione reductase and glutathione peroxidase activities of the liver are unaffected by ethanol administration. It is suggested that the decrease in reduced glutathione content may reflect a condition of increased peroxidation in vivo.

INTRODUCTION

Previous reports (1) showed that peroxidation of liver lipids, as measured in vitro by the formation of malonic dialdehyde (MDA) by liver homogenates, is greatly enhanced after acute ethanol intoxication. An increase in the formation of thiobarbituric acid (TBA) positive material also occurs following the addition of ethanol in vitro to liver homogenates (1). Both effects have been confirmed (2,3), but the mechanism by which ethanol stimulates the lipoperoxidation of liver preparations is still obscure.

However possible explanations for such an effect brought about by ethanol could be offered by the results of some early experimental reports: (a) the decrease in the lipid soluble antioxidant concentration in liver mitochondria following acute ethanol intoxication, found by Di Luzio and Hartman (4); (b) the enhanced NADPH oxidase activity found by Lieber and De Carli (5) in liver microsomes of rats fed alcohol for several days; (c) the lowered content of reduced glutathione (GSH) reported by Takada et al. (2) in whole liver of ethanol-treated rats; and (d) the changes in the redox state of the liver cell occurring as a consequence of ethanol metabolism (change in the NAD/NADH ratio and increase of the reduced pyridine coenzymes [6,7]). All these phenomena could affect the MDA production by liver homogenate, thus accounting for the enhanced peroxidation in vitro after ethanol intoxication; however in the former two instances the alteration would be located in the particulate cytoplasmic fractions, whereas in the latter cases the cause for the effect brought about by ethanol would be in the soluble supernatant. The present report describes experiments carried out with both isolated and recombined fractions and clearly shows that the factors by which ethanol stimulates the peroxidation of liver lipids are to be searched for in the soluble fraction of the liver cell.

MATERIALS AND METHODS

Female Sprague-Dawley rats (180-220 g), maintained on a pellet diet (Piccioni, Brescia, Italy) free of preservative compounds, were used. After a 14 hr fast, ethanol was given by oral intubation as a 50% solution, in a single dose of 6 g/kg. Control rats received glucose in a dose isocaloric to ethanol. In some experiments saline controls (receiving saline isovolumetric to ethanol) were also performed. All animals were starved after dosing, and killed 6 hr later unless otherwise stated.

In the experiments carried out with isolated fractions, either mitochondria or microsomes were separated from 10% liver homogenates in 0.25 M sucrose (8). The sedimented fractions were washed once in 0.15 M KCl and resuspended in the same medium to give a concentration of either mitochondria or microsomes equivalent to 100 mg liver per milliliter.

Since sucrose is known to interfere with the TBA reaction (9), liver homogenates were prepared in ice cold 0.15 M KCl, when the soluble supernatant was desidered in the incubation system. The microsomal plus supernatant fraction was obtained by centrifuging the homogenate at 9000 x g for 12 min (10). The "microsomal" fraction and the soluble supernatant were then obtained by centrifuging the 9000 x g supernatant at 95,000 x g for 60 min. The so-called mitochondrial fraction was obtained by removing the nuclei from the whole homogenate at 600 x g for 8 min and then by centrifuging the supernatant fluid at 9000 x g for 12 min. This fraction contained less than one-third of glucose-6-phosphatase activity with respect to that found in the "microsomal"

TABLE I

Malonic Dialdehyde Production by Various Liver Preparations and by Cross-Recombined Fractions of Liver from Glucose- and Ethanol-Treated Rats, 6 Hours after Ethanol Administration[a]

Treatment	Liver preparation	Malonic dialdehyde, mμmol/mg protein		Experiment no.
		Incubation time		
		60 min	120 min	
Saline	Homogenate	1.65 ± 0.10	2.97 ± 0.10	5
Glucose	Homogenate	1.72 ± 0.15	2.89 ± 0.11	5
Ethanol	Homogenate	2.86 ± 0.41[b,c]	4.22 ± 0.39[f,c]	5
Glucose	Microsomes	1.20 ± 0.13	2.28 ± 0.31	3
Ethanol	Microsomes	0.90 ± 0.14	2.26 ± 0.20	3
Glucose	Microsomes[e]	22.54 ± 1.25	28.37 ± 1.45	7
Ethanol	Microsomes[e]	20.37 ± 1.90	26.22 ± 2.39	7
Glucose	Mitochondria	1.01 ± 0.15	1.75 ± 0.21	3
Ethanol	Mitochondria	0.86 ± 0.09	1.82 ± 0.10	3
Glucose	Mitochondria[e]	14.88 ± 0.64	20.01 ± 0.58	3
Ethanol	Mitochondria[e]	16.02 ± 0.19	19.55 ± 0.39	3
Glucose	Supernatant	0.02	0.02	2
Ethanol	Supernatant	0.02	0.01	2
Glucose Glucose	Microsomes Supernatant	3.52 ± 0.13	5.91 ± 0.20	4
Ethanol Ethanol	Microsomes Supernatant	4.49 ± 0.50[d]	8.84 ± 1.22[d]	4
Glucose Glucose	Mitochondria Supernatant	2.14 ± 0.27	4.06 ± 0.38	6
Ethanol Ethanol	Mitochondria Supernatant	2.90 ± 0.22[d]	5.67 ± 0.38[b]	9
Glucose Ethanol	Supernatant Microsomes	3.36 ± 0.51	5.47 ± 0.99	3
Glucose Ethanol	Microsomes Supernatant	4.88 ± 0.23[b]	7.60 ± 0.24[d]	3
Glucose Ethanol	Supernatant Mitochondria	1.75 ± 0.22	3.55 ± 0.22	5
Glucose Ethanol	Mitochondria Supernatant	2.50 ± 0.13[f]	4.92 ± 0.42[d]	8

[a]Results represent mean ± SEM.

[b]$P<0.02$.

[c]Significantly different from both saline and glucose controls.

[d]$P<0.05$.

[e]0.4 μmol ascorbic acid added to incubation mixture.

[f]$P<0.01$.

fraction. The sediments were recombined with the soluble supernatant to give a concentration of fractions equivalent to 100 mg liver per milliliter.

The incubation system contained, in a final volume of 4.0 ml, 225 μmol of K-phosphate buffer, pH 7.4, and 2.5 ml of either the whole homogenate or the suspension of cytoplasmic fractions. In some of the experiments carried out with isolated fractions (without the soluble supernatant), 0.4 μmol of ascorbic acid was added to the incubation mixture. Incubation was carried out aerobically, at 37 C. The amount of MDA formed was determined as previously reported (1,11). (It has been previously demonstrated by one of us [1] that no MDA breakdown occurs under these experimental conditions and that the concentration of MDA in the medium at the end of incubation reflects MDA formation.)

Protein content of liver preparations was determined by the method of Lowry et al. (12).

The GSH content of the liver supernatant was determined by the alloxan "305" method (13).

NADPH oxidase activity was determined according to Gillette et al. (14).

Glutathione reductase and glutathione per-

TABLE II

NADPH Oxidase, Glutathione Reductase and Glutathione Peroxidase Activities of Liver, 6 Hours after Saline, Glucose or Ethanol Administration

Treatment	NADPH oxidase activity, mμmol NADPH oxidized/ min/mg protein[a]	GSSG reductase activity, mμmol GSSG reduced/ min/mg protein[b]	GSH peroxidase activity, mμmol GSSG formed/ min/mg protein[c]
Saline	5.2	36.2 ± 2.9	501 ± 36
Glucose	5.9	40.3 ± 2.9	537 ± 48
Ethanol	5.4	35.8 ± 2.0	575 ± 5

[a]Results are mean of two experiments carried out with three pooled livers.

[b]Results are mean of five experiments ± SEM.

[c]Results are mean of three experiments ± SEM.

oxidase activities were determined according to Pinto and Bartley (15). Since significant changes in the latter activity have been shown to occur during the oestrous cycle (16), the rats were taken in the same oestrous stage (metoestrous). The stage of the oestrous cycle was based on vaginal smears.

RESULTS AND DISCUSSION

In agreement with previous observations (1), liver homogenates from ethanol-treated rats formed a higher amount of MDA, compared to both glucose and saline controls, 6 hr after ethanol administration (Table I). The cause of the alteration does not seem to be located in either mitochondria or microsomes since these fractions, when isolated and incubated both in the presence and in the absence of ascorbic acid, did not form a higher amount of MDA in ethanol-treated animals with respect to that formed by the glucose controls (Table I). The soluble supernatant of the liver from either glucose- or ethanol-treated rats formed almost no MDA when incubated without the other subcellular fractions. Barber (17) also noted that the soluble supernatant alone does not produce MDA, whereas the presence of the supernatant is essential for MDA production by the other subcellular fractions.

When mitochondria or microsomes were recombined with their soluble supernatant, a greater MDA production was observed again in the ethanol group as compared to the controls (Table I). Therefore it seems likely that the cause of the ethanol-induced increase in the peroxidation of liver lipids is to be searched for in the soluble fraction of the liver cell. This conclusion is further supported by experiments in which either the "mitochondrial" or the "microsomal" fraction from the glucose controls was recombined with the soluble supernatant from the ethanol-treated rats and vice versa. In fact, the addition of the soluble supernatant from intoxicated animals to either the "mitochondrial" or the "microsomal" sediment from controls increased the MDA production well above the amount formed by the samples in which the supernatant from control animals and the "mitochondrial" or the "microsomal" sediment from ethanol-treated animals was present (Table I).

Therefore it seems that, in our system, the nature of either the "mitochondrial" or the "microsomal" fraction is not a critical factor for the extent of the peroxidation, which is, on the contrary, affected by the nature of the soluble fraction of the liver cell. Thus it appears untenable that the ethanol-induced increase in the lipoperoxidation of liver homogenates is due to a decrease in the lipid soluble antioxidant content of some cytoplasmic organelles, which occurs following ethanol intoxication (4). Rather the decrement could be caused by the enhanced peroxidation.

Lieber and De Carli (5) suggested that the peroxidation of liver lipids could be stimulated by the increased H_2O_2 production associated with the enhanced NADPH oxidase activity of liver microsomes in rats fed ethanol for several days. This hypothesis, although highly provocative, does not seem to apply to acute ethanol toxicity, since, as shown above, the effect brought about by acute ethanol administration on liver lipid peroxidation is bound to the soluble fraction and not to the microsomes of the liver cell. However, to check this point further, microsomes from either control (glucose or saline) or ethanol-treated rats were incubated in the appropriate conditions for detecting NADPH oxidase activity (14). It was found that the amount of MDA formed after 5, 15, 30, 60, 120 min of aerobic incubation was not different in the various groups of animals; nor did an increase in the NADPH oxidase activity occur in liver microsomes 6 hr after acute ethanol intoxication (Table II).

TABLE III

Malonic Dialdehyde Production by Microsomal Fraction of Liver Recombined with Supernatant Heated at 100 C in Glucose- or Ethanol-Treated Rats, 6 Hours after Dosing

Treatment	Liver preparation	Malonic dialdehyde, mμmol/mg protein	
		Incubation time	
		60 min	120 min
Glucose Glucose	Microsomes Supernatant	1.09	2.25
Ethanol Ethanol	Microsomes Supernatant	2.57	4.88
Glucose Glucose	Microsomes Heated supernatant	4.33	6.78
Ethanol Ethanol	Microsomes Heated Supernatant	3.99	6.62

Microsomes from both glucose- and ethanol-treated rats were also tested in the system used by May and McCay (18,19) for the enzymic peroxidation catalyzed by NADPH oxidase. No increase in MDA production was found in the ethanol group. Thus the enzyme system that controls the peroxidation of liver lipids (18-20) is not changed by acute ethanol intoxication. Nevertheless peroxidation could be affected by the ethanol-induced changes in the redox state of pyridine coenzymes (6,7), which results in an increased availability of NADPH in the cell sap.

In order to ascertain whether the factor in liver supernatant of ethanol-treated rats that is responsible for the increased peroxidation was heat labile, an experiment was carried out in which the liver supernatants from glucose- or ethanol-treated rats were heated at 100 C for 5 min and then recombined with their microsomal fractions. Difference in the MDA production between ethanol- or glucose-treated animals was seen no more (Table III). However these results are complicated by the fact that heating the soluble supernatant resulted in an increased MDA production, regardless of whether the animals had been ethanol- or glucose-treated.

The increased MDA production by liver from ethanol-treated rats cannot be related to the fat accumulation in the liver cell following alcohol administration, as suggested by others (3), since it was previously shown (1) that: (a) The increase in liver lipid peroxidation occurs prior to the increase in liver triglyceride concentration. (b) The peroxidation returns to normal values when the liver triglyceride level is at its maximum, i.e., 24 hr after ethanol feeding. (c) Ethanol stimulates the peroxidation, even when it is added in vitro to liver homogenates.

Takada et al. (2) found that the GSH content of the liver is decreased following acute

TABLE IV

Reduced Glutathione Content of Liver Supernatant and Malonic Dialdehyde Formation by Microsome plus Supernatant Fraction of Liver, 2 and 6 Hours after Glucose or Ethanol Administration[a]

Treatment	Hours after glucose or ethanol	GSH content, mμmol/mg protein	Malonic dialdehyde, mμmol/mg protein	
			Incubation time	
			60 min	120 min
Glucose	2	35.06 ± 1.38 (11)	1.94 ± 0.09 (6)	4.04 ± 0.23 (6)
Ethanol	2	31.05 ± 2.04[b] (11)	2.70 ± 0.08[c] (6)	5.40 ± 0.21[d] (6)
Glucose	6	36.18 ± 0.81 (6)	1.80 ± 0.06 (4)	3.98 ± 0.22 (4)
Ethanol	6	23.13 ± 0.65[c] (6)	2.46 ± 0.22[e] (4)	5.07 ± 0.43[e] (4)

[a]Results represent mean ± SEM. Number of experiments given in parentheses.

[b]Not significantly different from glucose controls.

[c]$P<0.001$.

[d]$P<0.002$.

[e]$P<0.05$.

ethanol intoxication. Since GSH inhibits lipid peroxidation in liver mitochondria and microsomes (21,22), a decrease in the GSH content of the soluble fraction of the liver could well give rise to an increase in MDA formation, thus explaining the enhanced peroxidation of liver preparations containing the supernatant from ethanol-treated rats. As shown in Table IV, the GSH content of the soluble fraction of the liver is significantly decreased 6 hr after ethanol administration, thus confirming the results of Takada et al. (2). However no significant decrease in GSH was seen 2 hr after the administration of alcohol; nevertheless, at this time, lipid peroxidation of the microsome plus supernatant fraction is already increased (Table IV). It therefore seems that the lowering in GSH is not the cause of the enhanced peroxidation in acute ethanol toxicity. Conversely the decrease in this low molecular weight thiol could reflect a condition of increased peroxidation in the liver cell, since it has been demonstrated that GSH inhibits lipid peroxidation of mitochondria and microsomes by a reaction with the peroxide intermediates and that the enzyme involved (glutathione peroxidase) catalyzes the oxidation of GSH by the fatty acid hydroperoxides (21-25).

This view is indirectly supported by the fact that no significant decrease in the glutathione reductase activity of the liver was found 6 hr after ethanol administration (Table II), and therefore the lowering in the hepatic GSH content cannot be referred to an ethanol-induced inhibition of the enzyme activity that regenerates GSH. Also, the glutathione peroxidase activity of the liver is not altered 6 (Table II) or 18 hr after ethanol administration.

Glutathione peroxidase is believed to play a physiological role in decomposing the hydroperoxides occurring in the normal living hepatocyte (23-25); thus the corresponding hydroxy acids would be formed in the liver cell and these compounds might be easily metabolized (23-25). On the basis of this assumption, we can speculate that ethanol stimulates the physiological route of the peroxidation of liver lipid, possibly because of the higher availability of NADPH in the cell sap. If the peroxides formed are quickly decomposed by the glutathione peroxidase system, the only change detectable in vivo (in such a condition direct evidence of peroxidized lipids has not been univocally obtained [26-29] and therefore the occurrence of lipid peroxidation in vivo after acute ethanol intoxication is questionable) is the decrease in the GSH content of the liver from ethanol-treated animals. The condition of enhanced peroxidation is thus displayed only by the system in vitro, in which the peroxidation starts after the concentration of low molecular weight thiols is decreased to low levels (21,22).

REFERENCES

1. Comporti, M., A. Hartman and N.R. Di Luzio, Lab. Invest. 16:616 (1967).
2. Takada, A., F. Ikegami, Y. Okumura, Y. Hasumura, R. Kanayama and J. Takeuchi, Ibid. 23:421 (1970).
3. Bunyan, J., M.A. Cawthorne, A.T. Diplock and J. Green, Brit. J. Nutr. 23:309 (1969).
4. Di Luzio, N.R., and A.D. Hartman, Exp. Mol. Pathol. 11:38 (1969).
5. Lieber, C.S., and L.M. De Carli, Science 170:78 (1970).
6. Cherrick, G.R., and C.M. Leevy, Biochim. Biophys. Acta 107:29 (1965).
7. Lieber, C.S., Annual Rev. Med. 18:35 (1967).
8. de Duve, C., B.C. Pressman, R. Gianetto, R. Wattiaux and F. Appelman, Biochem. J. 60:604 (1955).
9. Wilbur, K.M., F. Bernheim and O.W. Shapiro, Arch. Biochem. Biophys. 24:305 (1949).
10. Ghoshal, A.K., and R.O. Recknagel, Life Sci. 4:1521 (1965).
11. Comporti, M., C. Saccocci and M.U. Dianzani, Enzymologia 29:185 (1965).
12. Lowry, O.H., N.J. Rosembrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193:265 (1951).
13. Patterson, J.W., and A. Lazarow, in "Methods of Biochemical Analysis," Vol. II, Edited by D. Glick, Interscience Publishers, New York, 1955, p. 259.
14. Gillette, J.R., B.B. Brodie and B.N. LaDu, J. Pharmacol. Exp. Ther. 119:532 (1957).
15. Pinto, R.E., and W. Bartley, Biochem. J. 112:109 (1969).
16. Pinto, R.E., and W. Bartley, Ibid. 115:449 (1969).
17. Barber, A.A., Radiat. Res. (Suppl.) 3:33 (1963).
18. May, H.E., and P.B. McCay, J. Biol. Chem. 243:2288 (1968).
19. May, H.E., and P.B. McCay, Ibid. 243:2296 (1968).
20. Hochstein, P., and L. Ernster, Biochem. Biophys. Res. Commun. 12:388 (1963).
21. Christophersen, B.O., Biochem. J. 100:95 (1966).
22. Christophersen, B.O., Ibid. 106:515 (1968).
23. Christophersen, B.O., Biochim. Biophys. Acta 164:35 (1968).
24. Christophersen, B.O., Ibid. 176:463 (1969).
25. Little, C., and P.J. O'Brien, Biochem. Biophys. Res. Commun. 31:145 (1968).
26. Kalish, G.H., and N.R. Di Luzio, Science 152:1390 (1966).
27. Hashimoto, S., and R.O. Recknagel, Exp. Mol. Pathol. 8:225 (1968).
28. Di Luzio, N.R., Ibid. 8:394 (1968).
29. Comporti, M., E. Burdino and F. Raja, Life Sci. 10:855 (1971).

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Effect of Ethanol on Plasma Triglycerides in Male and Female Rats

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ABSTRACT

A single large dose of ethanol was given to fasted rats and to rats fed a fat-free diet containing orotic acid. An increased plasma triglyceride concentration after ethanol feeding was consistently found in fasted male rats, while the results in fasted female rats varied between the experiments. The total rate of triglyceride secretion into the plasma in fasted rats was estimated as the Triton-induced hypertriglyceridemia 6-7.5 hr after ethanol feeding. The effect of ethanol on the triglyceride secretion from extrahepatic sources was estimated in the same way in rats, with the hepatic triglyceride secretion blocked by orotic acid. Ethanol enhanced the Triton-induced hypertriglyceridemia in both male and female fasted rats, but to a greater extent in male rats. Ethanol did not stimulate the extrahepatic triglyceride secretion during this period.

INTRODUCTION

Hyperlipemia has often been observed in association with human alcoholism (1). Chronic feeding of ethanol to rats causes increased plasma triglyceride concentrations and an increased secretion of lipoproteins from the liver to the plasma (2).

Horning et al. (3) reported an increased plasma triglyceride concentration in both male and female rats 6 hr after a single large dose of ethanol, and that the increase was larger in male rats than in female rats. These authors suggested that this increase was due to an increased secretion of triglycerides from the liver to the plasma. However other reports suggest that ethanol does not influence the triglyceride secretion to the plasma and does not increase the concentration of plasma lipids (4,5). It has also been reported that ethanol may decrease the rate of triglyceride secretion from perfused rat livers (6).

Recently it was reported that the intestinal secretion of very low density lipoproteins increased after ethanol had been given intraduodenally to fasted rats, and it was suggested that this was a contributing factor to the ethanol-induced increase in the plasma triglyceride concentration (7).

The present investigation is an attempt to elucidate the effect of ethanol on the plasma triglyceride concentration in fasted male and female rats. We have previously studied the effects of ethanol on the removal rate of chylomicron triglycerides from the plasma (8). The present report concerns the effect of ethanol on the secretion rate of triglycerides into the plasma. The time course of the plasma triglyceride concentrations after a single large dose of ethanol was followed. The total secretion of triglyceride into the plasma was estimated as the Triton-induced hypertriglyceridemia, and the extrahepatic part of the secretion was estimated in the same way in rats in which the hepatic secretion of very low density lipoproteins was blocked by feeding a diet containing orotic acid. This was done to find out whether the sex difference in the plasma triglyceride concentration after ethanol could be explained by a difference in the rate of secretion of triglycerides to the plasma and, if so, to what extent the triglycerides were secreted from the liver or from the intestine.

MATERIALS AND METHODS

Injection Solution

Triton WR 1339 (Winthrop Labs., New York) was dissolved in 0.9% NaCl (w/v) to a 20% (w/v) solution.

Animal Procedure

Sprague-Dawley rats (Anticimex, Stockholm, Sweden) of the same age, males weighing ca. 170 g and females ca. 150 g, were used. They were kept in an artificially lighted room with the lights on 8 A.M.-8 P.M. Until the time of the experiments, the rats in experiments 1, 2 and 4 were fed a standard laboratory diet (AB Ewos, Södertälje, Sweden) ad libitum. In experiment 3 the rats were given either a fat-free semisynthetic diet containing 2% orotic acid or the same diet without orotic acid for 7 days ad libitum. The diets were essentially the same as those used by Windmueller and Levy (9). The major constituents of the diets were casein, 20%; glucose, 70.5%; vitamin-sucrose mixture (10), 5%; salt mixture (11), 4%; L-cystein, 0.3%; and choline chloride, 0.2%. The rats had

free access to water throughout the experiments.

Experimental Design

The unanesthetized rats were tube-fed ethanol 6 g/kg body wt as a 38% (v/v) solution in water or saline 20 ml/kg body wt (0.9% w/v NaCl). Tube-feeding of ethanol or saline was always done between 8 and 9 A.M., and consequently the triglyceride secretion was estimated at the same time of day in all experiments. In previous studies the control rats have been given a single dose of glucose isocaloric to that of ethanol (4,12). We preferred to give the control rats saline for the following reasons. A glucose dose isocaloric to the ethanol dose (6 g/kg body wt) used in the present study is probably metabolized within a few hours, while the ethanol is metabolized during a much longer time period. Thus the effects of glucose should be restricted to the first few hours after feeding. During these hours glucose either does not influence (12) or decreases (13) the plasma triglyceride concentration. Glucose also has several other effects on lipid metabolism that ethanol has not (8,12). To avoid possible difficulties in the interpretation of our results, we therefore decided to give our control rats saline and not glucose.

The rats in experiments 1, 2 and 4 were fasted for 24 hr before tube-feeding. In experiment 3 the diets were removed after tube-feeding. The rats in all experiments were killed by exsanguination via the aortic bifurcation under light ether anesthesia. In experiments 2 and 3 the rate of triglyceride secretion to the plasma was estimated as the Triton-induced hypertriglyceridemia. Triton WR 1339 is a nonionic detergent that blocks the removal of triglycerides from the plasma, probably without interfering with the secretion of triglycerides to the plasma (14,15). The Triton-induced hypertriglyceridemia may therefore be used to estimate the rate of triglyceride secretion to the plasma (15). In experiment 3 orotic acid was given in a fat-free diet as described under Animal Procedure. Orotic acid selectively blocks the hepatic secretion of very low density lipoproteins and, consequently, the major part of the triglyceride secretion from the liver to the plasma (16). The Triton-induced hypertriglyceridemia in rats treated in this way should be an estimation of the extrahepatic triglyceride secretion to the plasma (17). As the orotic acid-induced blockage of the hepatic triglyceride secretion is rapidly reversed when orotic acid is withdrawn (9,16), the rats were tube-fed ethanol or saline without prior fasting. The rats were anesthetized with ether 6 hr after tube-feeding and 0.7-1.0 ml blood was withdrawn from a neck vein. Triton WR 1339 (50 mg/100 g body wt) was then injected into the same vein. The animals were killed 90 min later. The blood was transferred to chilled heparinized centrifuged tubes and plasma was collected after centrifugation at +4 C. Plasma lipids were extracted with 20 volumes chloroform-methanol 2:1 v/v. In experiment 4 the liver was rapidly removed, rinsed in water, blotted dry, cut free of extrahepatic fat, weighed and homogenized in 20 volumes chloroform-methanol 2:1 v/v. Chloroform extracts of plasma and liver lipids were prepared according to Folch et al. (18).

Analyses

Lipid classes from liver and plasma were separated by thin layer chromatography on Silica Gel G (Merck, AG, Darmstadt, Germany). The plates were sprayed with 0.2% (w/v) 2,5-dichlorofluorescein in ethanol, the fractions located under UV light and the triglycerides scraped off into glass columns and eluted with chloroform. Glyceride-glycerol in the eluates was determined according to Carlson (19).

Statistics

Differences between groups of rats were tested using Wilcoxon's rank sum test. Differences were considered significant for p-values less than 0.05.

RESULTS

At 0 hr the plasma triglyceride concentration was higher in fasted male than in fasted female rats ($p<0.01$) (Table I). In the male rats the plasma triglyceride concentration increased between 0 and 6 hr and between 6 and 13 hr after ethanol feeding, and was significantly higher than in the corresponding control rats both at 6 and 13 hr. In the female rats the plasma triglyceride concentration increased in both the saline-fed and the ethanol-fed rats from 0 to 6 hr. In the ethanol-fed female rats the plasma triglyceride concentration continued to increase between 6 and 13 hr and at 13 hr was significantly higher than in the saline-fed controls. In experiment 2 (Table II) the plasma triglyceride concentration in the ethanol-fed female rats was significantly higher than in the controls already at 6 hr; but, also in this experiment, it was lower than in the ethanol-fed male rats ($p<0.05$) at 6 hr.

Triton induced a hypertriglyceridemia in all groups in experiment 2 (Table II). This hyper-

TABLE I

Effect of Ethanol on Plasma Triglyceride Concentrations in Fasted Male and Female Rats (Experiment 1)

Treatment	Time after treatment, hr	Plasma triglyceride concentration, μmol/ml[a] Male	Female
None	0	0.35 ± 0.03 (10)	0.25 ± 0.04 (10)
Saline	6	0.39 ± 0.05 (9)	0.41 ± 0.04 (9)
Ethanol	6	0.75 ± 0.09[b] (8)	0.45 ± 0.04[c] (10)
Saline	13	0.32 ± 0.03 (9)	0.31 ± 0.02 (10)
Ethanol	13	0.94 ± 0.08[b] (9)	0.73 ± 0.07[d] (10)

[a]Values are mean ± SE. Number of rats in parentheses. *p*-Values refer to differences between a group of rats given ethanol and the corresponding control group.

[b]$p<0.005$.

[c]$p>0.05$.

[d]$p<0.01$.

triglyceridemia was, however, smaller in both groups of male rats than in any of the groups of female rats (Table II). Thus the rate of triglyceride secretion to the plasma appears to be slower in the male than in the female rats in the fasting state. The Triton-induced hypertriglyceridemia was larger in the ethanol-fed than in the corresponding saline-fed rats (Table II). This difference between the ethanol-fed and the control rats was statistically significant in the male but not in the female rats, and was also larger in the male than in the female rats.

In experiment 3 orotic acid was given to block the hepatic secretion of very low density lipoproteins. Rats fed the control diet not containing orotic acid had higher plasma triglyceride concentrations both before and 90 min after the Triton injection than did the corresponding fasted rats in experiment 2 (fasted ethanol-fed with and without Triton) (Tables II and III). This is probably an effect of the high carbohydrate content of the control diet (20). In the rats given the control diet and tube-fed ethanol there was no sex difference in the plasma triglyceride concentration before injection of Triton (Table III). As in the other experiments the Triton-induced increase in plasma triglycerides was more pronounced in the female than in the male rats ($p<0.01$). In all groups fed a diet containing orotic acid the plasma triglyceride concentration was much lower both before and after injection of Triton, than in the rats fed a diet without orotic acid (Table III). This indicates that treatment with orotic acid was effective and shows the predominant role of the liver as a source of plasma triglycerides (17,21). When the rats fed an orotic acid-containing diet and ethanol are compared to the rats fed the same diet and saline, it appears that the ethanol-fed male rats had a significantly smaller Triton-induced increase in the plasma triglyceride concentration than their saline-fed controls, while this increase was smaller but not significantly smaller in the ethanol-fed than in the saline-fed female rats (Table III). Thus ethanol did not stimulate the extrahepatic secretion between 6 and 7.5 hr under these experimental conditions. It is also shown (Table III) that the plasma triglyceride concentration before and 90 min after Triton injection was not sex-dependent ($p>0.05$) when the rats had been fed an orotic acid-containing diet. Thus there appears to be no sex difference in the rate of extrahepatic triglyceride secretion.

TABLE II

Effect of Ethanol on Triton-Induced Hypertriglyceridemia in Fasted Male and Female Rats (Experiment 2)

Treatment	Plasma triglyceride concentration, μmol/ml[a] Male	Female
Before injection of Triton		
Saline	0.41 ± 0.05 (6)	0.30 ± 0.03 (5)
Ethanol	0.86 ± 0.03 (6)[b]	0.62 ± 0.07 (6)[b]
90 min after injection of Triton		
Saline	3.19 ± 0.29	5.87 ± 0.41
Ethanol	4.89 ± 0.18	6.92 ± 0.44
Triton-induced increase		
Saline	2.78 ± 0.26	5.56 ± 0.39
Ethanol	4.03 ± 0.19[b]	6.30 ± 0.45[c]

[a]Values are mean ± SE. Number of rats in parentheses. *p*-Values refer to differences between a group of rats given ethanol and the corresponding control group. Triton WR 1339 was injected iv 6 hr after ethanol feeding.

[b]$p<0.005$.

[c]$p>0.05$.

TABLE III

Effect of Ethanol on Triton-Induced Hypertriglyceridemia in Male and Female Rats Given an Orotic Acid-Containing Diet (Experiment 3)

Treatment	Plasma triglyceride concentration, μmol/ml[a]	
	Male	Female
Before injection of Triton		
Orotic acid diet + saline	0.05 ± 0.01 (7)	0.06 ± 0.02 (5)
Orotic acid diet + ethanol	0.06 ± 0.02 (7)	0.09 ± 0.03 (5)
Control diet + ethanol	1.04 ± 0.07 (7)	0.97 ± 0.28 (5)
90 min after injection of Triton		
Orotic acid diet + saline	1.54 ± 0.10	1.47 ± 0.26
Orotic acid diet + ethanol	1.21 ± 0.11	1.02 ± 0.11
Control diet + ethanol	6.89 ± 0.36	8.63 ± 0.48
Triton-induced increase		
Orotic acid diet + saline	1.49 ± 0.11	1.41 ± 0.27
Orotic acid diet + ethanol	1.17 ± 0.11[b]	0.93 ± 0.09
Control diet + ethanol	5.85 ± 0.31	7.66 ± 0.46

[a]Values are mean ± SE. Number of rats in parentheses. *p*-Values refer to differences between a group of rats given ethanol and the corresponding saline-fed control group. Triton WR 1339 was injected iv 6 hr after ethanol feeding.

[b]$p<0.05$.

DISCUSSION

In male rats ethanol feeding increased the plasma triglyceride concentration both in these experiments and in other experiments under identical conditions (O. Johnson and O. Hernell, unpublished). This effect of ethanol on the plasma triglyceride concentration in male rats has also been a consistent finding in experiments by others (3,12). In contrast, results with female rats have varied (3-5,22). In one of our previous (unpublished) experiments the plasma triglyceride concentrations did not increase during the first 16 hr in fasted female rats given ethanol. In another experiment the plasma triglyceride concentration was not increased after 6 hr, but increased from 0.23 ± 0.03 to 1.41 ± 0.31 μmol/ml (values are mean ± SE for six rats) between 6 and 13 hr. Other investigators also found no such increase in female rats after ethanol feeding (4,5). Horning et al. (3) observed an increase in plasma triglycerides 7-8 hr after ethanol feeding in fasted female rats, but the increase in different experiments varied between 50 and 300 μg/ml. It thus appears that, when female rats develop an increased plasma triglyceride concentration as a response to ethanol feeding, they often do so after a lag period of several hours. This may explain some of the divergent results previously reported (3-5,22). However there is also considerable variation in results from apparently similar experiments. It may be that the exact nutritional and hormonal states are very important. Watkins et al. (23) found marked effects of estrogen on the output of triglycerides from perfused livers derived from ovariectomized female rats. Thus the estrous cycle in female rats may influence the plasma triglyceride levels via the liver triglyceride secretion. It has also been shown (14) that in fed female rats the time of the day may influence the triglyceride secretion into the plasma.

The mechanisms responsible for the ethanol-induced increase in plasma triglyceride concentration must be an increased rate of triglyceride secretion to the plasma or a decreased removal of triglycerides from the plasma or both. It has previously been shown that ethanol feeding does not alter the removal of triglycerides following the iv injection of chylomicra, if the experiment is designed to avoid the interference of increased concentrations of endogenous lipoproteins (2,8). The results in the Triton experiment (Table II) suggest that ethanol feeding stimulates the secretion of triglycerides to the plasma, and this seems to be the mechanism responsible for the ethanol-induced increase in plasma triglyceride concentration, at least in male rats.

The liver is the main source of the plasma triglycerides when resorption of exogenous fat does not take place (17,21). However the intestine secretes triglycerides in very low density lipoproteins, even during periods with no resorption of exogenous fat (16,17). Mistilis and Ockner (7) infused ethanol intraduodenally in male rats and observed an increased concentration of triglycerides in the intestinal mucosa followed by an increased secretion of triglycerides into the intestinal lymph. The present results from Triton experiments in rats with blocked hepatic secretion of very low density lipoproteins indicate that ethanol feeding caused an inhibition of the extrahepatic triglyceride secretion to the plasma in the male and probably in the female rats between 6 and 7.5 hr after ethanol feeding (Table III). Thus the ethanol-induced increase in the plasma triglyceride concentrations (in fasted rats) at this time, i.e., 6 hr, was probably due to increased secretion of triglycerides from the liver rather than from the intestine to the plasma. As a consequence, the hypothesis (6) that the accumulation of triglycerides in the liver after a

single large dose of ethanol is caused by a decreased secretion of triglycerides from the liver to the plasma appears less likely.

A consistent finding in these experiments was that the triglyceride secretion into the plasma was more rapid in female than in male rats. This confirms a previous report (14). The present results show that this is due to a sex difference in the hepatic triglyceride secretion, since there appears to be no sex difference in the extrahepatic triglyceride secretion (Table III). In agreement with this, Watkins et al. (23), in liver perfusion experiments, found a more rapid release of triglycerides from livers derived from female than from livers derived from male rats. Thus the triglyceride secretion to the plasma is quantitatively a more important factor in the liver lipid metabolism in female than in male rats.

Watkins et al. (23) found similar plasma triglyceride concentrations in male and female rats, in spite of a more rapid release of triglycerides from perfused livers derived from female than from livers derived from male rats. They concluded that it might be necessary to postulate that the utilization of very low density lipoproteins by extrahepatic tissues is slower in male than in female rats. Our results support this, since in the ethanol-fed males a secretion of triglycerides to the plasma, which is even slower than in the saline-fed females with low plasma triglyceride concentration, leads to a high plasma triglyceride concentration (Tables I and II). Furthermore, though the rate of triglyceride secretion to the plasma is higher in the female than in the male saline-fed rats (Table II), there is not a higher plasma triglyceride concentration in the female rats (Tables I and II).

Ethionine, orotic acid and cycloheximide induce a fatty liver mainly by a common mechanism, i.e., an almost complete blockage of the triglyceride secretion from the liver to the plasma (24-26). An observation in fatty livers induced by any of these substances is that female rats have a larger increase in liver triglycerides than male rats (24-26). Part of the explanation may be that the triglyceride secretion from the liver—the parameter affected—is faster in female than in male rats in the normal state (14) (Table II), and thus is quantitatively more important in the liver lipid metabolism of female rats. Also in the acute ethanol-induced fatty liver there is a larger increase in the liver triglycerides in female rats compared to male rats (27). In a separate experiment (experiment 4) the liver triglyceride concentration increased between 0 and 6 hr in the female rats from 13.0 ± 2.0 to 27.3 ± 4.4 and in the male rats from 6.6 ± 0.6 to 11.4 ± 1.6 μmol/g liver (values are mean ± SE for five or six rats). However under these conditions the liver triglyceride secretion is increased rather than decreased. The cause of the liver lipid accumulation lies in some other parameter (28). There may well be a sex difference also in this parameter. In addition, however, the increased secretion of triglycerides from the liver, which is more pronounced in the male rats, helps limit the liver lipid accumulation. The sex difference in the liver triglyceride accumulation can therefore be explained, at least in part, by the larger ethanol-induced increase in the liver triglyceride secretion in the male rats compared to the female rats.

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REFERENCES

1. Lieber, C.S. Ann. Rev. Med. 18:35 (1967).
2. Baraona, E., and C.S. Lieber, J. Clin. Invest. 49:769 (1970).
3. Horning, M.G., M. Wakabayashi and H.M. Maling, in "Biochemical Factors in Alcoholism," Edited by R.P. Maicker, Pergamon Press, Oxford, 1967, p. 139.
4. DiLuzio, N.R., Life Sci. 4:1373 (1965).
5. Seakins, A., and D.S. Robinson, Biochem. J. 92:308 (1964).
6. Schapiro, R.H., G.D. Drummey, Y. Shimizu and K.J. Isselbacher, J. Clin. Invest. 43:1338 (1964).
7. Mistilis, S.P., and R.K. Ockner, J. Lab. Clin. Med. 80:34 (1972).
8. Olivecrona, T., O. Hernell, O. Johnson, G. Fex, L. Wallinder and O. Sandgren, Quarterly J. Stud. Alcohol. 33:1 (1972).
9. Windmueller, H.G., and R.I. Levy, J. Biol. Chem. 242:2246 (1967).
10. Sidransky, H., and S. Clark, Arch. Pathol. 72:468 (1961).
11. Hegstedt, D.M., R.C. Mills, C.A. Elvehjem and E.B. Hart, J. Biol. Chem. 138:459 (1941).
12. Ylikahri, R.H., Metabolism 19:1036 (1970).
13. Baker, N., A.S. Garfinkel and M.C. Schotz, J. Lipid Res. 9:1 (1968).
14. Otway, S., and D.S. Robinson, J. Physiol. 190:321 (1967).
15. Otway, S., and D.S. Robinson, Ibid. 190:309 (1967).
16. Windmueller, H.G., R.I. Levy and A.E. Spaeth, Adv. Exp. Med. Biol. 4:365 (1969).
17. Windmueller, H.G., and R.I. Levy, J. Biol. Chem. 243:4878 (1968).
18. Folch, J., M. Lees and G.H. Sloane-Stanley, Ibid. 226:497 (1957).
19. Carlson, L.A., J. Atheroscler. Res. 3:334 (1963).
20. Eaton, R.P., and D.M. Kipnis, Amer. J. Physiol. 217:1153 (1969).
21. Havel, R.J., J.M. Felts and C.M. Van Duyne, J. Lipid Res. 3:297 (1962).
22. Elko, E.E., W.R. Wooles and N.R. DiLuzio, Amer.

J. Physiol. 201:923 (1961).
23. Watkins, M.L., N. Fizette and M. Heimberg, Biochim. Biophys. Acta 280:82 (1972).
24. Farber, E., in "Advances in Lipid Research," Vol. 5, Edited by R. Paoletti and D. Kritchevsky, Academic Press, London, 1967, p. 119.
25. Sidransky, H., Endocrinology 72:709 (1963).
26. Bar-on, H., O. Stein and Y. Stein, Biochim. Biophys. Acta 270:444 (1972).
27. Mallow, S., and J.L. Block, Amer. J. Physiol. 184:29 (1956).
28. Johnson, O., O. Hernell, G. Fex and T. Olivecrona, Life Sci. 10:553 (1971).

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Distribution of *trans*-6-Hexadecenoic Acid, 7-Methyl-7-Hexadecenoic Acid and Common Fatty Acids in Lipids of the Ocean Sunfish *Mola mola*

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ABSTRACT

Lipids extracted from various tissues of four individual sunfish have been shown to contain the *trans*-6-hexadecenoic acid previously reported for marine turtles, a metridium and a jelly fish, and also the 7-methyl-7-hexadecenoic acid recently isolated from one sunfish liver oil sample. The other fatty acids present were qualitatively typical of marine lipids in general. Unusual quantitative details included high percentages of 18:0 and 20:4ω6, which are also found in the Atlantic leatherback turtle and presumably linked to a similar diet of jellyfish and to other common factors. In a sample of lipids from intestinal contents *trans*-6-hexadecenoic acid was found to be the predominant C_{16} monoene, and was accompanied by comparatively large amounts of the 7-methyl-7-hexadecenoic acid. This observation and other fatty acid details are compatible with an exogenous origin for these acids and jellyfish, etc., as a predominant dietary material for the ocean sunfish.

INTRODUCTION

Jellyfish have been reported to be dietary factors common to the leatherback turtle *Dermochelys coriacea coriacea* (L) and the ocean sunfish *Mola mola* (L) (1,2). Our interest in marine food chains led us to carry out an analysis of lipids from a single specimen of sunfish caught in the summer of 1969 off the Nova Scotia coast. Three additional sunfish were caught in the summer of 1971 to confirm our early results, because of unnusual features of fatty acids and the dearth of accurate biological data. Thin layer chromatographic (TLC) and gas liquid chromatographic analysis of these lipids for *trans*-6-hexadecenoic acid (*t*-16:1ω10) led to the discovery of 7-methyl-7-hexadecenoic acid (7-M-16:1ω9) as a significant and unusual component, which was isolated for study from liver lipid (3). (The shorthand notation of chain length: number of double bonds and position of the double bond relative to the terminal methyl group is conveniently supplemented with the prefixes *t* for *trans* and 7-M for 7-methyl.)

With the benefit of hindsight, the 7-M-16:1ω9 acid probably also appears as a minor ($<<1\%$) component in the fats of marine turtles (4), a metridium (5), a jellyfish (6) and perhaps in other analyses where it has been reported as tentatively identified 16:2ω6, which would usually fall in about the same position.

In one of these fish the intestinal content was successfully extracted and the presence of both unusual fatty acids lends credence to our belief that their origin is dietary for the sunfish and probably for the leatherback turtle (4).

EXPERIMENTAL PROCEDURES

Samples and Lipid Recovery

Sunfish 1969-1 was caught in a tuna trap off Queensland, N.S., on July 25, 1969, transported to the Halifax Laboratory of the Fisheries Research Board, and frozen the same day. This specimen weighed ca. 60 kg and was male. On August 4th the fish was dissected after being allowed to thaw overnight. Tissue samples were taken as follows: the white collagenous subdermal material (WCSM), 2-5 cm in thickness in our specimens; a thin "fatty" fibrous layer found between this layer and the muscle; white muscle; dark muscle; and a portion of the liver.

All 1971 sunfish were female with no overt gonad development. Details were: Sunfish 1971-1 weighing 190 kg, was caught off Crouchers Island, N.S., on July 2, 1971. It was dissected in the field the same day. Samples of the liver and white muscle were analyzed for fatty acid content. Sunfish 1971-2 was caught off Eastern Passage, N.S., July 29, 1971, and weighed 90 kg. Samples of white muscle and liver were taken. Sunfish 1971-3 was caught in St. Margaret's Bay and was taken to the F.R.B. facilities at Boutiliers Point Biological Station on August 18, 1971. Total weight was ca. 150 kg. Samples obtained included liver and intestinal contents.

Sunfish 1969-1

The lipids were extracted by the method of

Bligh and Dyer (7) and, with the exception of those from the thick white collagenous layer were separated on a scale of 100-500 mg into phospholipids, triglycerides and "other" or on a 25 x 400 mm column of Dow styrene-divinylbenzenecopolymer beads (X_2, 200-400 mesh) eluted with benzene (8,9). Esters were prepared by transesterification of these fractions using 7% BF_3-methanol and refluxing for 30-45 min. The total lipids from the WCSM were saponified following AOCS Method Ca-6b-53. The recovered fatty acids were converted to methyl esters by refluxing briefly with BF_3-methanol.

Sunfish 1971-1,2,3

The lipids from the flesh and liver samples were extracted by the method of Bligh and Dyer (7), and the flesh lipids separated into phospholipid and triglyceride fractions on the gel column. The liver lipids, a clear yellow oil in each case, were found by TLC to be mainly triglyceride. These were purified by gel chromatography, and saponified by AOCS Method Ca-6b-53. The fatty acids were recovered and converted to methyl esters by refluxing with 7% BF_3-methanol.

The intestinal contents were a milky fluid. Lipid was extracted by shaking with petroleum ether. The emulsion formed was centrifuged and the clear upper layer decanted and dried over Na_2SO_4. TLC of the recovered lipid showed small amounts of phospholipids, free sterol, triglycerides and sterol esters, and a large amount of free fatty acid. The sample was saponified as above and the fatty acids purified by preparative scale TLC on Prekotes (Adsorbosil 5, from Applied Science Lab.) eluted by hexanes-diethyl ether-acetic acid 70:30:1. The recovered acids were esterified as described for sunfish 1969-1.

Analyses of samples

GLC analyses of all esters were carried out on 150 ft open-tubular capillary columns coated with butanediolsuccinate polyester (BDS) in Perkin-Elmer Model 226, 900 or 990 GLC apparatus as previously described (4,10). The presence of *t*-16:1ω10 acid was confirmed by preparative scale TLC on silver nitrate-silica plates (Supelcosil 12D) developed with *n*-hexane-benzene 1:1. Analyses of both total esters and the monoene fractions from TLC were carried out on Apiezon columns to confirm the presence of the two unusual acids (11). Quantitation based on Disc Instruments Inc. integrators may have had total errors of the order of ±5% for major (>10%) components, ±10% for moderate scale (1-9%) components, or as much as ±30% for minor (<1%) components. A portion of each ester sample was hydrogenated and analyzed qualitatively and quantitatively as a check on correction factors in the total analysis. The presence of 7-methyl-7-hexadecenoic acid in the 1971 sunfish had previously been demonstrated in detail (3).

RESULTS AND DISCUSSION

The first sunfish examined (1969-1) had a rather lean liver compared to the sunfish livers extracted in 1971 (Table I). However the liver oils of all four fish when fractionated on the gel column were found to be >95% triglyceride. The first fraction to elute, ~2%, was considered to be phospholipids, and TLC showed the remaining 2% to be made up of free fatty acids and sterol esters with the properties of cholesterol esters.

The dark muscle lipid on TLC examination contained a large spot for free fatty acids. It may have been partially hydrolyzed during frozen storage (9), and the lipid fatty acid results are not included in Table I. Similarly the results of the phospholipid analysis of the liver have been omitted as possibly misleading as to their original fatty acid compositions. These two tissues exhibit more enzymatic activity towards lipids than the white muscle. The fatty acids for dark muscle total lipid were basically similar in composition to the white muscle triglyceride results of Table II. The so-called "fibrous layer" was initially thought to be a fatty membrane layer between the WCSM and white muscle, but this proved rather lean (0.3% lipid, of which 38% was recovered as triglycerides and 49% as phospholipid). The WCSM referred to above is the white collagenous layer between 2 and 5 cm in thickness, which surrounds the visceral organs but has no obvious function (12). This material emulsified severely during the Bligh and Dyer extraction and the $CHCl_3$ layer yielded only 0.02% lipid. TLC indicated mostly polar lipids. The low proportion of triglyceride in this lipid (Table I) suggests that the lipid is natural to this tissue and not contamination from adjacent tissue. However the lipid may have been localized in nerves or other specialized inclusions rather than part of the bulk of the tissue. Both chemical and histological analyses confirmed that this material was mainly collagen (D.H. Shaw, P.H. Odense, private communications).

The fatty acid analyses of the lipids from the various sunfish tissues (Table II) include all the major and minor fatty acids found in lipids of other higher marine species that we have analyzed, e.g., cod liver oil (10), and the two unusual fatty acids previously reported (3,4).

TABLE I

Lipid Recoveries from Samples and from Gel Chromatography of Lipid Extracts

Sunfish	Sample	% Lipid	% Triglycerides	% Phospholipids	% "Other"
Sunfish 1969-1	Liver, 4.2% of fish	31.9	96.2	1.8	1.5
	Dark flesh	1.21	42.7	51.2	8.6
	White flesh	0.48	32.8	59.5	8.2
	Fibrous Layer	0.30	38	49.2	12.4
	White collagenous layer	.02	<10	>80	∿10
Sunfish 1971-1	Liver, 3.2% of fish	44.3	>95	>2	∿3
	White flesh	0.73	58	20.8	N.D.
Sunfish 1971-2	Liver, 5.0% of fish	56.0	>95	>2	∿3
	White flesh	0.22	36	60	N.D.
Sunfish 1971-3	Liver	42.0	>95	<2	∿3
	Intestinal content	N.D.	>75% free fatty acids remainder phospholipids, mono-, di- and tri-glycerides.		

The percentages of *t*-16:1ω10 and 7-M-16:1ω9 show a parallel relationship in all samples. For example in the four liver oils (Table II), the ratios *t*-16:1ω10 to 7-M-16:1ω9 were 1.12, 1.20, 1.12 and 1.27. In the intestinal content and WCSM, where both values are unusually high, these ratios are 1.05 and 1.26, respectively. This constant value for the two unusual fatty acids in four different fish indicates that these are both normal components for the ocean sunfish, and as the *t*-16:1ω10 is thought to be exogenous in origin the same origin may be inferred for the 7-M-16:1ω9.

An unusual feature in two of the four individuals (sunfish 1969-1 and sunfish 1971-3) is the ratio in all lipids of 22:1ω11 + 13<22:1ω9. The normal proportion for marine oils containing these acids is 22:1ω11 + 13>22:1ω9. This peculiarity in two animals is observed not only in the liver oil, where these depot-type acids are important, but also in the rest of the lipids, including phospholipids. Other monoethylenic acids do not show a comparable distribution of related isomers. However 20:1ω7 nearly equal to 20:1ω9 in 1969-1 and 1971-3 is also novel, and the proportion of 20:1ω7 in the 20:1 isomers is higher than is found in depot fats of most higher marine animals, e.g., 4,10,13-16, except data in some analyses of marine turtles, where parallels may be found (4). Our jellyfish studies (6,8) provide clear evidence for 20:1ω7 > 20:1ω9 and 22:1ω9 > 22:1ω11 + 13.

The most common fatty acids (Table II) have some interesting features. For example the saturated acids show the minor component distribution pattern usual for marine lipids from higher animals; but 18:0 in the liver oil is undoubtedly at least twice the percentage usually found in marine animal triglycerides, although not exceptional for phospholipids. In the opinion of the authors, 20:0 is also a more obvious component in the sunfish liver oils, perhaps aobut twice the percentage commonly observed in other fish depot fats; but less importance should be attached to this because of the possible errors in GLC quantitation of a component of this magnitude. The saturated acids as percentages of totals in the four liver oils are quite surprisingly uniform at 31, 31, 28 and 30%, whereas the total usually observed in marine oils is 20-25% of fatty acids.

Considering the size of the animal, the thickness of the integument and the propensity for basking on the ocean surface, the level of 18:0, etc., in the liver oil might suggest an internal body temperature above ambient such as observed (17) in the leatherback turtle, an animal of comparable size and behavior pattern. The iodine values calculated for the fatty acids of the white muscle phospholipids, 168 and 188 (Table II), are quite low, as 200-220 would be expected for fish muscle phospholipid fatty acids (18). One interpretation would be to associate this also with a higher body temperature, but comparative data for fish muscle are lacking. The presence of 20:4ω6 in lieu of 20:5ω3 or 22:6ω3 accounts for only 5-10 IV units of this difference, which otherwise is due to generally low levels (25-30%) for the total of 20:5ω3 + 22:6ω3. This total is usually 35-45% in Atlantic fish muscle phospholipids, and it would seem possible that the proportions of the ω3 acids in the ocean sunfish phospholipids reflect functional differences, while the ω6 acids reflect dietary influences.

Among the polyunsaturated fatty acids of the liver oils, the ω6 or linoleic group is unusually important for marine fish depot fats, but mostly in the form of 20:4ω6—another

TABLE II

Weight Per Cent Composition[a] of Fatty Acids in Various Lipids Recovered from Four Specimens of Ocean Sunfish *Mola mola* and Jellyfish *Cyanea capillata*[a]

	Mola mola Specimen, tissue and lipid														
	1969-1					1971-1			1971-2			1971-3			
	Liver-isolated	White muscle	Fibrous layer		WCSM	Liver oil	White muscle		Liver oil	White muscle		Liver oil	Intestine contents	Jelly fish	
Fatty acid	TG	TG	TG	PL	Total	Total	TG	PL	Total	TG	PL	Total	Total	TG	Polar
12:0	Trace	Trace	ND	Trace	ND	Trace	Trace	.69	Trace	ND	ND	Trace	ND	0.17	ND
14:0	2.00	.75	1.32	.48	4.55	4.72	.94	1.25	3.59	.15	.74	2.18	.86	1.92	0.91
4,8,12	.07	.03	ND	.03	.16	ND	.04	ND	ND	.03	ND	ND	ND	0.11	ND
Iso 15:0	.10	.05	.27	.03	.32	.33	.05	.10	.06	.05	.05	.22	.15	0.56	0.30
Anteiso 15:0	.03	.01	.05	.02	.16	.02	.02	.06	.02	0.2	.01	.09	.08	0.11	0.06
15:0	.73	.45	.55	.30	1.93	.86	.31	.25	.68	.39	.37	.65	.66	1.07	0.84
Iso 16:0	.08	.08	.05	1.06	.64	.12	.04	.20	.13	.05	.05	.13	.09	0.33	0.12
Pristanic	ND	.10	.05	ND	ND	.04	.17	.10	ND	ND	.13	ND	ND	0.11	1.43
16:0	16.49	15.50	14.47	14.35	22.77	16.52	12.71	15.74	14.42	15.46	13.42	15.82	7.76	13.32	6.86
Iso 17:0	.66	.10	.30	.17	.06	.57	.70	.20	.38	.11	.13	.47	.72	1.10	ND
Anteiso 17:0	.16	.10	.09	.06	Trace	.10	.09	.16	.13	.06	.09	.17	.12	0.99	0.35
17:0	.64	1.14	.75	.87	1.42	1.06	.59	.71	1.08	.67	.63	.76	.88	0.55	0.47
Phytanic	.09	.05	.21	.14	ND	.16	.12	.02	.12	.12	.09	ND	ND		
Iso 18:0	.22	.22	.13	.03	.12	.15	.19	.25	.35	.35	.18	.08	.12	0.44	0.12
18:0	8.88	14.40	14.19	14.53	11.55	6.48	9.97	12.51	6.22	8.97	11.03	9.22	6.59	4.57	4.39
19:0	ND	.54	.44	.46	.49	.14	.35	.41	.28	.41	.48	.21	.25	0.65	0.86
20:0	.35	.44	.65	.14	.55	.22	.30	.32	.15	.28	.20	.31	.10	1.40	2.41
Total[b]	31	34	34	33	45	31	27	33	28	27	28	30	19	27	19
14:1ω9?	—	.26	.66	.09	.81	.21	—	—	.21	.08	.05	—	.06	ND	ND
14:1ω7?	.07	—	—	—	—	—	3.24	.10	.39	.23	.07	.13	.06	ND	ND
15:1	.03	.38	.11	ND	.53	.02	.09	.05	.02	.12	.25	ND	ND	ND	ND
t-16:1ω10	2.74	1.62	1.28	1.16	3.45	2.94	1.05	.71	2.48	.77	.72	2.96	3.84	2.22	0.89
16:1ω7	5.50	2.02	1.63	1.40	3.00	6.87	2.38	2.02	6.16	1.53	1.80	5.08	1.83	1.11	0.15
16:1ω5	.11	.15	ND	.23	ND	.33	.17	.10	.38	.64	.27	.13	.20	0.14	ND
7-Methyl-7-hexadecenoic	2.44	2.12	1.09	2.45	2.81	2.45	1.33	1.01	2.22	1.24	1.35	2.33	3.67	1.20(?)	0.38(?)
17:1ω10	ND	ND	ND	ND	ND	.12	.03	.03	.09	.09	.09	ND	ND	ND	ND
17:1ω8	.95	.50	.32	.17	.40	.49	.43	.33	.50	.32	.27	.42	.70	0.08	0.03
18:1ω9	15.70	10.29	9.65	7.99	7.96	11.39	12.61	14.09	8.83	8.88	10.03	17.64	3.48	5.17	1.04
18:1ω7	3.77	2.38	3.15	2.28	1.73	2.31	2.74	1.79	2.12	1.75	1.16	2.49	1.75	1.94	0.98
18:1ω5	.32	.05	.21	0.05	.06	.16	.27	.20	.54	.19	.20	.57	.36	ND	0.22
19:1	.28	.20	.05	.06	.07	.20	.08	.05	.19	.06	.10	.10	.05	0.05	ND

20:1ω11	.21	.10	.13	0.03	.12	.36	.34	.39	.26	.43	.65	.21	.04	2.56	1.33
20:1ω9	2.42	2.01	3.27	.80	1.22	4.17	4.58	2.90	7.54	4.61	3.27	3.80	.93		
20:1ω7	1.08	.71	1.32	.23	.98	1.55	1.15	.49	.86	.34	.52	2.36	.66	3.28	4.70
20:1ω5	.11	.02	.05	.06	.12	.08	.08	.05	.12	.05	.04	.26	.05	0.64	1.14
22:1ω13+11	.45	.92	1.41	ND	.61	2.32	4.36	.97	5.61	7.35	2.29	1.42	.09	0.53	0.06
22:1ω9	.58	1.19	1.93	ND	.30	1.18	2.01	.15	1.40	.98	.34	2.29	.36	2.90	0.68
22:1ω7	.08	.10	.05	ND	ND	.04	.07	ND	.30	.06	ND	.18	.01	0.42	0.14
22:1ω5	ND	ND	ND	ND	ND	.13	ND	ND	ND	ND	ND	ND	ND	0.08	ND
24:1	.05	.12	.20	ND	ND	.02	.50	ND	ND	.33	.09	ND	.11	ND	ND
Total[c]	37	25	26	18	25	37	38	25	40	29	22	42	18	22	12
16:2	ND	ND	ND	ND	ND	ND	.07	ND	.25	.02	ND	ND	ND	0.11	ND
18:2ω6	.83	.94	.57	.74	.93	.80	.82	1.09	1.12	1.14	1.36	.93	1.99	0.81	0.17
20:2ω6	.51	.22	.41	.26	.12	.24	.20	.17	.49	.21	.26	.97	.91	1.33	1.08
22:2ω6	.13	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.42	0.22
Total	1.5	1.2	1.0	1.0	1.0	1.0	1.1	1.3	1.9	1.4	1.6	1.9	2.9		
18:3ω6	.09	.15	ND	.09	.03	.23	.28	ND	.20	ND	ND	ND	ND	0.08	ND
18:3ω3	.60	.05	.16	.38	.25	ND	.31	.06	.11	.50	.31	.75	.72	0.51	0.12
20:3ω6	ND	.05	.05	ND	ND	ND	ND	ND	ND	ND	ND	ND	.30	ND	ND
20:3ω3	.19	.08	.06	ND	ND	.04	.06	ND	.15	ND	.08	.29	.40	0.65	0.28
Total	.88	.33	.27	.47	.28	.27	.65	.06	.46	.50	.39	1.04	1.42	1.24	.40
16:4ω1	ND	ND	ND	.31	ND	.04	.07	.40	.06	ND	ND	ND	ND	0.11	ND
18:4ω3	.61	.27	.29	.03	.19	2.96	.48	.69	3.09	.78	.62	1.09	.48	0.84	0.13
20:4ω6	3.13	7.73	5.93	10.62	10.30	1.35	3.80	6.21	1.74	5.89	7.35	1.79	9.26	3.27	11.10
20:4ω3	.57	.15	.12	.03	.25	.51	.21	.21	.52	.13	.23	.62	.45	0.92	0.61
22:4ω6	.65	.61	.18	.54	.14	.11	.24	.42	.10	.17	.42	.26	.60	0.46	1.64
Total	5.0	8.8	6.5	11.5	10.9	5.0	4.8	7.9	5.5	7.0	8.6	3.8	10.8	5.6	13.5
20:5ω3	7.24	8.67	5.70	6.90	3.77	7.70	8.34	10.47	7.55	9.31	11.01	7.12	17.41	12.38	24.46
21:5ω2?	.15	ND	ND	ND	ND	ND	ND	ND	.25	.07	ND	ND	.49	0.16	0.53
22:5ω6	.65	1.08	1.24	2.11	1.53	.26	.63	.76	.82	2.05	2.31	.64	1.40	1.34	2.15
22:5ω3	4.17	1.62	2.69	1.69	.72	5.02	3.81	2.52	3.06	1.37	1.15	3.90	2.81	3.99	5.51
Total	12.2	11.4	9.6	10.3	6.0	13.0	12.8	13.8	11.7	12.8	14.5	11.7	22.1	17.9	32.6
22:6ω3	10.87	16.97	21.60	21.55	8.22	10.72	16.16	14.70	11.16	18.75	18.30	8.75	24.48	21.15	17.56
Unknowns[d]	1.76	2.19	.99	5.18	3.75	1.17	.41	3.89	1.44	2.40	5.43	.21	2.62	2.00	3.50
Calculated IV	153	175	180	194	122	147	171	168	150	183	188	136	251	208	269

[a]TG = triglyceride. PL = polar lipid. ND = not determined usually for technical reasons associated with gas liquid chromatographic conditions. Two decimal places are for minor component comparison purposes only.

[b]21:1 and 22:0 were not determined, but 22:0 was not important.

[c]Two isomers were sometimes observed for 15:1, three for 17:1 and three for 19:1.

[d]Mostly a few corresponding to acetal breakdown products.

TABLE III

Chain Length Percentages of Triglyceride Fatty Acids as Determined on Hydrogenated Samples

	1969-1			1971-1		1971-2		1971-3
Fatty acid	Liver TG	White muscle TG	Fibrous layer TG	Liver oil TG	White muscle TG	Liver TG	White muscle TG	Liver TG
14:0	1.8	.5	.8	5.7	.9	5.1	1.4	2.3
16:0	26.5	25.9	17.8	24.4	20.0	26.2	19.9	25.6
18:0	28.6	23.9	28.1	23.7	27.1	23.5	23.8	30.8
20:0	11.7	17.9	17.5	20.7	17.6	19.3	24.7	16.6
22:0	18.2	23.1	29.6	25.9	29.5	21.0	21.8	17.5

feature shared with leatherback turtle oils (4) and also potentially linked to jellyfish (6,8). (In Table I of Reference 6, for 20:1ω6 read 20:4ω6.) The 22:5ω6 is also unusually high in these oils, but may be linked to the fact that 22:5ω3 is nearly half of 22:6ω3 rather than to the accumulation of 20:4ω6. In our experience with fish liver and depot oils, a 1:10 or 1:5 ratio for 22:5ω3 and 22:6ω3 would be normal. The possible biochemistry of higher proportions of 22:5ω3 in marine mammal oils has been discussed elsewhere (15). Thus, in the $C_{22} > C_{20}$ relationship for total chain lengths (Table III), there is an indication of conditions favoring chain extension to the C_{22} polyethylenic acids, but not necessarily 22:6ω3.

Table III summarizes the overall compositional relationships among the four samples of liver triglycerides and also suggests that the white muscle triglycerides, with the exception of the C_{14} acids, resemble the corresponding liver triglyceride in fatty acid composition. In some fatty acids (1971-1, 1971-2) there are indications that the muscle triglycerides are also related to the phospholipids in composition. For example percentages for 18:0, 20:4ω6, 20:5ω3 and 22:5ω3 are intermediate (Table II). This type of organ or tissue specific association has been noted elsewhere, i.e., in seal lipids (19).

The white muscle phospholipids had fatty acid compositions that were slightly abnormal. The total for the linolenic series acids 20:5ω3 + 22:5ω3 + 22:6ω3 is usually 35-45% in comparable muscle phospholipids recovered from freshly killed marine fish or from those samples kept under ideal frozen storage conditions for short periods (9,14,16,18,20). The totals of these acids for sunfish 1971-1 and 1971-2 were 27.9% and 30.7%, but inclusion of the linoleic series acids 20:4ω6 and 22:5ω6 raises these totals to 35.3% and 40.8%, respectively, acceptable as normal values. This indicates that the two linoleic acids replace the acids of the linolenic acid series and are not extra to a normal percentage of the latter. Confirmation that these analyses are valid representations of original compositions is provided by an analysis carried out by J.C. Nevenzel (see also below), which gave 43% of C_{20} and C_{22} polyunsaturated fatty acids from total muscle phospholipids. From the total lipid these phospholipids were estimated as 22.6 ± 4.7% phosphatidylcholine and 9.5 ± 0.1% phosphatidylethanolamine. These proportions are generally observed in fish muscle (20). The four sunfish studied were all dead when examined by laboratory staff and adverse sample handling, including prolonged thawing of a whole fish (1969-1) or frozen storage of other samples (1971), would favor lipid hydrolysis (9,21,22). This process especially degrades phospholipids, not always equally (23), although this may not be obvious from liberated fatty acids (21,22). Triglycerides are also affected and may account for the low lipid recovery for white muscle sample 1971-2 (Table I). We believe that the fatty acid compositions of Table II for phospholipids from white muscle are possibly from mildly hydrolyzed phospholipids but are substantially correct.

The respective fatty acid compositions of the two lipids from the "fibrous layer" (Table II) do not show features that could be paralleled exactly in other isolated lipids. This type of layer may be a fascia layer, as indicated by the tough fibres observed and by the low lipid content. The superficial appearance of fat was evidently misleading, and other types of soft lubricious tissue may have been present. The WCSM, with virtually no recoverable lipid, cannot be discussed in detail, but the low proportions of 20:1 and 22:1 acids indicate that the lipid is cellular or membrane in origin (compare phospholipids). The IV of 122 calculated from the GLC analysis of total lipid methyl esters of fatty acids was the lowest of any tissue examined (Table II). Proportions of *t*-16:1ω10 and 7-M-16:1ω9 acid were unusually high, with *t*-16:1ω10 the predominant C_{16} monoene. These acids may have been associated with similar high proportions of 14:0 and 16:0

for reasons of similarity of chain length and physical behavior.

The concentration of fat in the liver of the ocean sunfish is not unusual. The common cod, *Gaduas morhua*, also has mostly white muscle containing ca. 0.75% lipid and the fat reserves are concentrated in the liver, which is 2-4% of the weight of the fish and 30-60% fat. Moreover the normal IV of 150-160 for cod liver oil is comparable to that of the *Mola mola* liver oils. Unlike the cod, the ocean sunfish has no swim bladder (24), but there is no evidence from the type of lipid in the liver that lipid is a factor in controlling buoyancy.

The habit of the ocean sunfish of basking on the ocean surface and some folklore of therapeutic properties of the liver oil in topical administration to man and animals led us to consider the vitamins in this oil. The Laboratory of the Government Chemist, London, U.K., examined the liver oil from specimen 1972-2 and found 180 μg/g of α-tocopherol but only traces of vitamin D. We are therefore inclined to discount that any such properties for this oil could be due to fatty acids or oil-soluble vitamins. However relatively short chain monoethylenic fatty acids are alleged to have therapeutic properties (25), and unknown properties might be conferred by 2-3% of *t*-16:1ω10 or 7-M-16:1ω9. Our iodine values for liver oil of 136-153 compare with 151.8, and unsaponifiable content of 1-3% with 3.2%, for oil from a Pacific specimen (26).

Gel chromatography of the lipid from the white muscle of fish 1969-1 yielded 8.2% of "other" lipids. There were not investigated in detail but the refractometer trace indicates that free sterol and sterol ester were important components. An older report (26) gives 24% unsaponifiable material in muscle lipid of iodine value 102.7 (presumably badly oxidized). This report led J.C. Nevenzel (private communication) to examine the unsaponifiables in the muscle lipid from a small Pacific *Mola mola*. The lipid included 14% free sterols (92% cholesterol by GLC), traces of hydrocarbons and a few per cent of probable wax esters. Otherwise the lipid sample composition was 30-40% phospholipid, 8% triglycerides and 23% free fatty acid (see Table I and discussion above).

The diet of the ocean sunfish includes shallow water bottom invertebrates, (27) but otherwise is fairly obscure. In addition to jellyfish (1,2,27), deep sea fish (of which some probably move near the surface at night) and seaweeds have been mentioned. Our examination of two fish showed empty stomachs and only a milky, oily looking fluid in the intestine. The latter, however, contained very little lipid. This lipid contained fatty acids (Table II) with very low levels of 16:1 (compare jellyfish [8] at 2.2-5.2%), and 18:1 (compare 4.5-7.2%); and high levels of 20:4ω6 (compare 6.2-9.1%), 20:5ω3 (compare 9.8-18.4%) and 22:6ω3 (compare 10.4-19.4%). We therefore feel that this analysis further supports our views linking jellyfish with the diet of the ocean sunfish. Observations by fishermen in Nova Scotian waters have indicated that the fish are active on the surface. Many are caught in net traps set for mackerel, herring or cod, suggesting a considerable degree of activity necessary to follow the net leader wall to the trap. The net may in fact, at the time of year usual for sunfish, also have large jellyfish entangled in it. Otherwise, sea gooseberries (*ctenophores*) are often common in the area near Halifax where ocean sunfish are observed. The suggestion that captures such as ours were moribund and therefore abnormal animals (24) may be discounted on grounds of the availability of food, the relatively warm water, and the apparently good liver condition in three fish.

Our study of fatty acids suggests that the ocean sunfish obtains a major part of its sustenance from jellyfish and similar pelagic invertebrates, which concentrate fatty acids of the linoleic acid type. Two unusual fatty acids may be of exogenous origin. There is a possibility that a fatty acid composition normal for marine fish muscle is modified by above-ambient internal body temperatures.

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REFERENCES

1. MacGinitie, G.E., and N. MacGinitie, "Natural History of Marine Animals," Second edition, McGraw-Hill, New York, 1968, p. 435.
2. Bleakney, J.S., Can. Field Naturalist 79:120 (1967).
3. Ackman, R.G., L. Safe, S.N. Hooper, M. Paradis and S. Safe, Lipids 8:21 (1973).
4. Ackman, R.G., S.N. Hooper and W. Frair, Comp. Biochem. Physiol. 40B:931 (1971).
5. Hooper, S.N., and R.G. Ackman, Lipids 6:341 (1971).
6. Hooper, S.N., and R.G. Ackman, Ibid. 7:624 (1972).
7. Bligh, E.G., and W.J. Dyer, Can. J. Biochem. Physiol. 37:911 (1959).
8. Sipos, J.C., and R.G. Ackman, J. Fish. Res. Bd. Can. 25:1561 (1968).
9. Drozdowski, B., and R.G. Ackman, JAOCS 46:371 (1969).
10. Ackman, R.G., J.C. Sipos and P.M. Jangaard, Lipids 2:251 (1967).

11. Hooper, S.N., and R.G. Ackman, Ibid. 5:288 (1970).
12. Rosen, N., Arkiv Zool. 8(10):1 (1913-14).
13. Ackman, R.G., and C.H. Castell, Lipids 1:341 (1966).
14. Ackman, R.G., and C.A. Eaton, Can. Inst. Food Tech. J. 4:169 (1971).
15. Ackman, R.G., S. Epstein and C.A. Eaton, Comp. Biochem. Physiol. 40B:683 (1971).
16. Ackman, R.G., and C.A. Eaton, J. Fish. Res. Bd. Can. 28:601 (1971).
17. Frair, W., R.G. Ackman and N. Mrosovsky, Science 177:791 (1972).
18. Ackman, R.G., JAOCS 43:385 (1966).
19. Ackman, R.G., S.N. Hooper and J. Hingley, Can. J. Biochem. 50:833 (1972).
20. Addison, R.F., R.G. Ackman and J. Hingley, J. Fish. Res. Bd. Can. 25:2083 (1968).
21. Ackman, R.G., P.J. Ke, W.A. MacCallum and D.R. Adams, Ibid. 26:2037 (1969).
22. Addison, R.F., R.G. Ackman and J. Hingley, Ibid. 26:1577 (1969).
23. Bligh, E.G., and M.A. Scott, Ibid. 23:1025 (1966).
24. Raven, H.C., Bull. Amer. Mus. Nat. Hist. LXXVI:143 (1939-40).
25. Grimmer, G., J. Kracht and R. Tschesche, Naturwissenschaften 48:718 (1961).
26. Kaufmann, H.P., and T. Miyakawa, Fette Seifen Anstrichm. 60:469 (1958).
27. Leim, A.H., and W.B. Scott, "Fishes of the Atlantic Coast of Canada," Fisheries Research Board of Canada Bulletin 155, 1966, 485 p.

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Sphingolipid Fatty Acid Composition of Rat Brain "Membranous Sacs"

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ABSTRACT

A fraction enriched in "membranous sacs" was isolated from the brains of young rats. Ceramide, glucosylceramide, galactosylceramide, lactosylceramide, sulfatide, sphingomyelin and ganglioside were purified. The fatty acids of the individual compounds were identified after hydrolysis. Certain similarities and dissimilarities were observed.

The interrelationship of several postulated intermediates of a metabolic pathway can occasionally be deduced from the detailed structural analysis of the naturally occurring compounds. A particulate fraction, originally described by Cleland and Kennedy (1) has been found to catalyze several of the reactions of sphingolipid biosynthesis in vitro (2). Electron microscopic examination indicated that this fraction contained a population of "membranous sacs" (2).

These particles have the capacity to catalyze most of the biosynthetic reactions in the absence of an exogenous source of sphingolipid acceptor (2). This paper describes the isolation and fatty acid composition of purified ceramide, glucosyl- and galactosylceramide, lactosylceramide, sulfatide, sphingomyelin and ganglioside from these preparations.

SPHINGOLIPID PURIFICATION

Particles were isolated from the brains of ca. 60 14 to 15-day-old Sprague-Dawley rats as previously described (3). The final pellet was suspended in distilled water, lyophilized, and the lipid extraction performed according to the procedure of Vanier et al. (4); then the mixture was grossly separated into ganglioside and nonganglioside lipids on a silicic acid column

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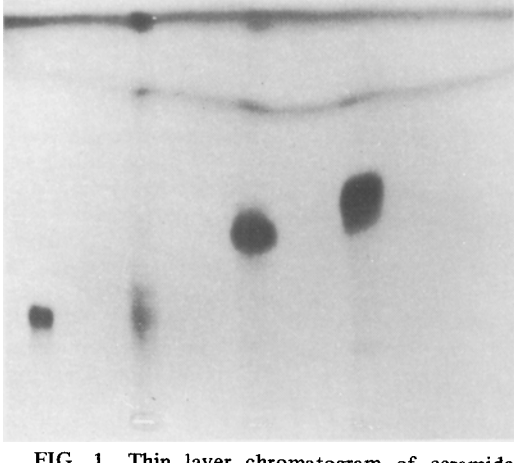

FIG. 1. Thin layer chromatogram of ceramide fractions isolated from rat brain particles (lanes from left to right): lane 1, HFA ceramide standard; lane 2, isolated HFA samples; lane 3, isolated NFA sample; lane 4, NFA ceramide standard. Solvent system chloroform-methanol-glacial acetic acid 90:2:8, spots revealed by charring.

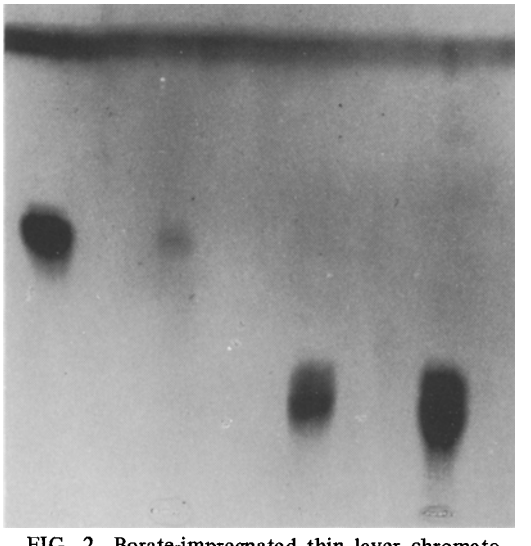

FIG. 2. Borate-impregnated thin layer chromatogram of cerebroside fractions isolated from rat brain particles (lanes from left to right): lane 1, glucosylceramide standard; lane 2, isolated glucosylceramide sample; lane 3, isolated galactosylceramide sample; lane 4, galactosylceramide standard. Solvent system chloroform-methanol-water 65:25:4, spots revealed by charring.

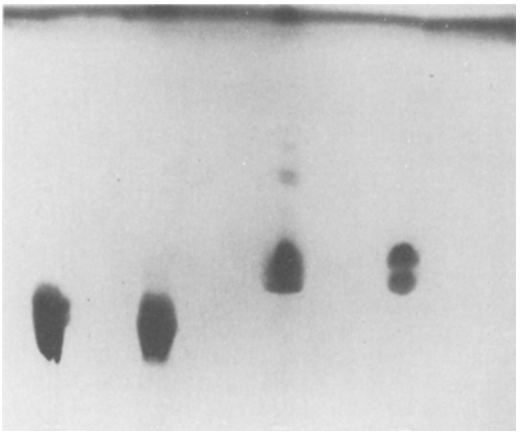

FIG. 3. Thin layer chromatogram of sphingomyelin and sulfatide fractions isolated from rat brain particles (lanes from right to left); lane 1, sphingomyelin standard; lane 2, isolated sphingomyelin sample; lane 3, isolated sulfatide sample; lane 4, sulfatide standard. Solvent system chloroform-methanol-water 65:25:4, spots revealed by charring.

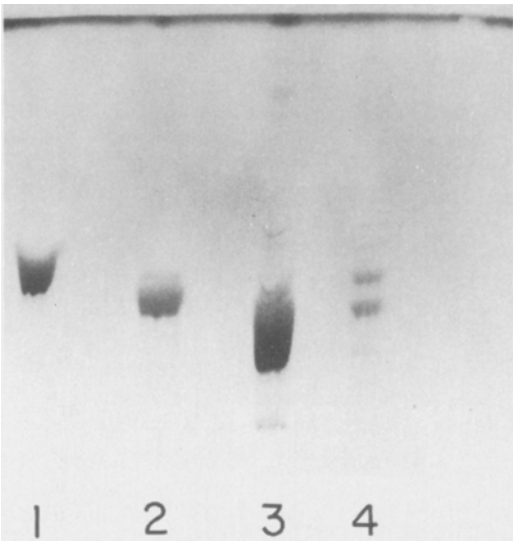

FIG. 4. Thin layer chromatogram of G_{Dla} fraction isolated from rat brain particles. Lane 1, G_{MI} standard; lane 2, isolated G_{Dla} sample; lane 3, G_{Dlb} standard (from R. McCluer); lane 4, mixed brain ganglioside standards. Solvent system chloroform-methanol-2.5N NH_4OH, spots visualized by charring.

(5). The ganglioside fraction was concentrated to dryness and subjected to preparative thin layer chromatography with Analtech Silica Gel G plates and chloroform-methanol-2.5N NH_4OH 60:35:8 as solvent. The band cochromatograming with G_{Dla} was removed, transferred to a small column and eluted with chloroform-methanol-water 60:35:8; this eluate was used without further purification for fatty acid analysis.

The nonganglioside lipid sample was taken to dryness and fractionated on a silicic acid (Unisil, Clarkson Chemical Co., Pa.) column into neutral lipids, sphingoglycolipids and phospholipids according to the method of Vance and Sweeley (6). The methanol eluate containing the phospholipids was subjected both to saponification and mercuric chloride-glacial acetic acid hydrolysis (7). The chloroform soluble material from this treatment was further purified by preparative thin layer chromatography. The band cochromatograming with sphingomyelin standard was scraped, transferred to a small column and eluted with chloroform-methanol-water 60:35:8 and used without further purification for fatty acid analysis.

The acetone eluate from the silicic acid column was concentrated to dryness and subjected to preparative thin layer chromatography. Bands corresponding to ceramide, sulfatide, cerebroside and lactosylceramide standards were removed, transferred to small columns and eluted with chloroform-methanol-water 60:35:8. The cerebroside fraction was subjected to further preparative thin layer chromatography on borate-impregnated Silica Gel G in order to separate the galactosyl and glycosyl derivatives with chloroform-methanol-water 65:25:4 as the solvent (8). Bands corresponding to glucosylceramide and galactosylceramide were well separated, scraped and transferred to small columns and eluted with chloroform-methanol-water 60:35:8; these separate eluates were used without further purification for fatty acid analysis. The material at the solvent front, which cochromatogramed with ceramide standard, was subjected to further preparative thin layer chromatography with chloroform-methanol-glacial acetic acid 90:2:8 as the solvent. Areas corresponding to both hydroxy and nonhydroxy fatty acid ceramide standards were transferred to small column and eluted with chloroform-methanol-water 60:35:8; these separate eluates were used without further purification for fatty acid analysis.

Aliquots of the purified samples were methanolyzed to obtain fatty acid methyl esters, methyl glycosides and sphingosine bases as previously described (9). The separation of nonhydroxy and hydroxy fatty acid methyl esters and the preparation of trimethyl silyl derivatives of hydroxy fatty acid methyl esters, methyl glycosides and sphingosine bases were performed according to Nagai and Kanfer (9).

Gas liquid chromatographic analyses were

carried out in a Hewlett-Packard 7620A Research chromatography using a flame ionization detector and connected to an electronic integrator capable of printing out retention times and relative peak areas. The fatty acid methyl esters were separated on a column containing 25% diethylene glycol succinate (DEGS) (Applied Science Lab., Inc.) coated on Chromasorb W (80-100 mesh, AW, DMCS; Applied Science Lab., Inc.). The nonhydroxy acids were analyzed at 180 C and hydroxy acids at 190 C. The identity of the peaks was also confirmed by analysis on 3% OV-1 column with temperature programing at the rate of 1 C/min. The analysis of nonhydroxy acids methyl esters was performed at 170-220 C and that of the hydroxy acids methyl esters at 180-230 C.

The analyses of trimethylsilyl derivatives of methyl glycosides and sphingosine bases were also carried out on the 3% OV-1 column used for the analysis of fatty acid methyl esters. The column was initially operated at 160 C for 8 min to complete the separation of sugars present in the sample, after which the temperature was raised to 200 C at the rate of 10 C/min and held constant for the separation of sphingosine bases.

RESULTS

The chromatographic purity of the final individual sphingolipid preparation is presented in Figures 1-4. It is apparent that little, if any, hydroxy fatty acid ceramides were obtained from these brain preparations (Fig. 1). The glucosyl and galactosyl ceramides were well separated (Fig. 2), and gas liquid chromatographic analysis of the carbohydrates after methanolysis indicated only the expected sugar as being present. G_{D1a} was the major ganglioside present although smaller quantities of other species were visible, and the material eluted from the preparative plate gave a single spot (Fig. 4). The sphingomyelin and sulfatides prepared from these particles appeared to be uncontaminated (Fig. 3). A trace of sulfatide appeared to be present in the lactosylceramide fraction. The fatty acid nonhydroxy and hydroxy composition of these purified sphingolipid samples is presented in Tables I and II. Sphingosine to dihydrosphingosine ratios of ca. 9:1 were obtained with all samples.

DISCUSSION

The ceramide fraction recovered from these particles contained only nonhydroxy fatty acids; however hydroxy fatty acids were present in both the galactosylceramide and sulfatide fractions. Carbohydrate analysis indicated that

TABLE I

Distribution of Nonhydroxy Fatty Acids in Individual Sphingolipids (wt %)

Nonhydroxy	Ceramide	Galactosylceramide	Sulfatide	Sphingomyelin	Glucosylceramide	Lactosylceramide	Ganglioside
C_{16}	2.9	0.6	3.2	5.4	1.2	9.4	2.9
C_{17}	0.2	---	---	0.4	---	---	---
C_{18}	87.2	19.9	39.8	84	61.0	43.0	93.3
$C_{18:1}$	Trace	---	1.0	Trace	0.6	1.8	Trace
C_{20}	4.2	5.8	6.6	3.6	7.5	9.4	3.8
C_{22}	1.8	20.8	12.7	2.3	12.0	12.3	---
$C_{22:1}$	Trace	---	0.4	---	Trace	---	---
C_{23}	0.3	3.7	1.8	0.3	1.5	1.2	---
C_{24}	1.8	40	24.6	2.2	15.2	17.4	---
$C_{24:1}$	1.6	7.7	8.4	1.7	0.9	4.1	---
C_{25}	---	0.7	0.2	---	---	---	---
C_{26}	---	1.0	1.2	---	---	1.4	---

TABLE II

Distribution of Hydroxy Fatty Acids in Individual Sphingolipids (wt %)

HFA	Galactosylceramide	Sulfatide	Lactosylceramide
C_{18}	5.4	24.5	14.8
C_{20}	2.8	12.7	5.0
C_{21}	—	0.7	—
C_{22}	38.7	26.0	40.9
$C_{22:1}$	—	5.4	4.6
C_{23}	3.1	1.8	—
C_{24}	47.1	18.5	34.8
$C_{24:1}$	Trace	5.3	—
$C_{25:1}$	1.0	—	—
$C_{26:1}$	2.0	5.1	—

glucosylceramide and galactosylceramide were pure and uncontaminated by one another. The lactosylceramide fractions were found to have an excess of galactose over glucose and possessed some hydroxy fatty acids suggesting a sulfatide contamination.

The ganglioside fraction, as reported by others (10), contains principally C_{18} with small amounts of C_{16} and C_{20} fatty acids. It is assumed that both glycosyl- and lactosylceramide are metabolic precursors of the gangliosides. The presence of relatively large amounts of the long chain fatty acids C_{22}-C_{24} in these simple sphingoglycolipids would tend to cast doubt upon the simplicity of this assumption and emphasize the need for substrate selectivity at some latter step in the sequence of reaction.

The nonhydroxyl fatty acid containing ceramides appears to reflect the complete spectrum of chain length present in the sphingolipids, with the C_{18} type being predominant. There is a close similarity in the fatty acid pattern of the ceramide and the sphingomyelin. The transfer of choline from CDP choline to the primary hydroxyl group of sphingosine, either free or N-acylated, is an area of great confusion. It is tempting to speculate that the ceramides containing predominantly C_{18} NFA are the preferred substrates for this reaction. The presence of ceramide in the cerebral particle systems could be due to its being actively produced in order to function as a precursor of the more complex sphingolipids, or to its being a catabolic product formed as a result of hydrolytic enzyme activities. The striking differences between the nonhydroxy fatty acid ceramide, glucosylceramide and gangliosides are difficult to explain. These two sphingolipids are presumed precursors of ganglioside, and the ceramide, a glucosylceramide precursor (10). In vivo studies, in which sphingosine-^{3}H was administered intercerebrally, revealed the labeling of only ceramide, sphingomyelin and ganglioside (11,12). The nonhydroxy fatty acids of these three sphingolipids are very similar and reinforce the metabolic interrelationship of this triad.

The enzymatic level at which a specific ceramide species becomes incorporated into intact sphingolipids is open to conjecture. Attempts to examine these specificities on the in vitro level have been undertaken only recently (13). Galactolipids can be formed by a series of reactions involving a psychosine intermediate. The N-acylation of galactosylsphingosine (14) would then select for the longer chain nonhydroxy and hydroxy fatty acids characteristic of these compounds. However the recent report of this acylation's being nonenzymatic has cast doubt concerning this reaction (15).

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REFERENCES

1. Cleland, W.W., and E.P. Kennedy, J. Biol. Chem. 235:45 (1960).
2. Kanfer, J.N., Chem. Phys. Lipids 5:159 (1970).
3. Kanfer, J.N. Lipids 4:163 (1969).
4. Vanier, M.T., M. Holm, R. Ohman and L. Svennerholm, J. Neurochem. 18:581 (1971).
5. Holm, M.R., Ibid. 15:821 (1968).
6. Vance, D.E., and C.C. Sweeley, J. Lipid Res. 8:621 (1967).
7. Abramson, M.B., W.T. Norton and R. Katzman, J. Biol. Chem. 240:2389 (1965).
8. Young, O., and J.N. Kanfer, J. Chromatogr. 19:611 (1965).
9. Nagai, Y., and J.N. Kanfer, J. Lipid Res. 12:143 (1971).
10. Svennerholm, L., in "Comprehensive Biochemistry," Vol. 18, Edited by M. Florkin and E.H. Stotz, Elsevier Publishing Co., Amsterdam, 1970, p. 201.

11. Kanfer, J.N., and A.E. Gal, Biochem. Biophys. Res. Commun. 22:442 (1966).
12. Kanfer, J.N., R.N. Bradley and A.E. Gal, J. Neurochem. 14:1095 (1967).
13. Radin, N.S., Adv. Exp. Med. Biol. 19:475 (1972).
14. Brady, R.O., J. Biol. Chem. 237:2416 (1962).
15. Hammerstrom, S., FEBS Lett. 21:259 (1972).

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Effect of Biliary Obstruction on Canine Plasma and Biliary Lipids

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ABSTRACT

The common bile duct was obstructed in 17 dogs. Reciprocal changes were noted for the plasma and biliary lipid concentrations of each after obstruction. As the plasma lecithin and free cholesterol concentrations increased, the biliary lipid concentrations declined. After biliary obstruction the reflux of biliary lecithin into the plasma of these animals was demonstrated with both labeled and unlabeled lecithin. The plasma lipid abnormalities seen after acute biliary obstruction were closely simulated by the reflux of lecithin alone from the biliary tree. The isolated reflux of biliary tract taurocholate produced a distinct lowering of plasma phospholipid and cholesterol concentrations, quite different from the plasma lipid alteration noted with acute biliary obstruction. Similar to observations in human obstruction, some of the plasma lipid was in mesophase form after these animals were obstructed.

INTRODUCTION

Obstruction of the biliary tree is associated with elevated plasma phospholipid and free cholesterol concentrations in a number of species (1-3) including man. It has recently been observed that in humans with biliary obstruction this phospholipid and sterol has a unique structure (4,5), quite different from conventional lipoproteins. Some of the plasma lipid has a liquid crystalline configuration similar to lipid structures in bile (6). The similarity in structure between this biliary lipid and that in plasma during obstruction suggests that the plasma lipid arises as a consequence of biliary reflux. However patients with lecithin cholesterol acyl transferase defects, who most probably have no net biliary to plasma lipid reflux, have plasma lecithin and free cholesterol in similar liquid crystalline form (7). Rather than an indication of its biliary origin, this plasma lipid configuration may merely reflect plasma-free cholesterol and lecithin levels in excess of the lipoprotein binding capacity for these lipids.

Previous studies (8,9) have suggested that the reflux of bile acid into the plasma of biliary obstructed rats was primarily responsible for the observed increases in plasma-free cholesterol and lecithin concentrations. It was necessary to infuse very large amounts of free cholic acid into the plasma of these animals to obtain a modest increase in their plasma phospholipid and cholesterol contents. Only free cholic acid, and not its conjugate, could initiate the plasma lipid alterations (9). Since unconjugated cholic acid is found in plasma in relatively minor amounts in obstruction (10), the relevance of this proposed sequence of events to the plasma lipid alterations in biliary obstruction is in some doubt.

The present study evaluates the pathogenesis of the plasma lipid increments that occur after biliary obstruction in dogs and the properties of the unusual plasma lipid forms seen with this disorder. The plasma and biliary lipid concentrations were studied sequentially after acute obstruction. To reproduce the plasma lipid pattern noted after acute obstruction two of the major canine biliary lipids, lecithin and taurocholate, were introduced into the chronically obstructed biliary system. With increases in only the lecithin concentrations of the obstructed biliary tract, both the plasma lecithin and free cholesterol concentrations rose in a manner similar to the plasma lipid alterations observed with acute obstruction. An increase in taurocholate concentration in the chronically obstructed tract had an opposite effect on the plasma lipids, producing a decrease in the concentration of both lecithin and cholesterol.

MATERIALS AND METHODS

Animals

Seventeen mongrel dogs were used for the studies. They ranged in weight from 10-23 kg and were maintained at the Duke University animal care facility and fed regular chow. Each dog was anesthetized with a single intravenous injection of pentobarbital (5 mg/kg body wt), prior to surgery. A cholecystectomy was performed through an abdominal incision, and a catheter placed in the common duct near its entrance to the duodenum. The common duct was ligated around the catheter and the catheter tunneled under the skin and exteriorized on

the anterior abdominal wall. The catheter end was plugged and sequential bile samples of less than 2 cc were periodically obtained from it, usually at biweekly intervals. Oral ampicillin (300-700 mg) was administered to the dogs throughout the study, and when biliary cultures demonstrated definite infection the study was terminated. A blood sample was obtained by femoral vein puncture each time a bile sample was collected. Blood was collected in disodium EDTA (1 mg/ml whole blood), and both blood and bile were maintained at 4 C after collection. When lipids were introduced into the obstructed biliary system an equal volume of bile was removed so that the biliary pressure, determined by mercury manometry, remained unchanged. The dogs were studied for periods up to 2 months. After sacrificing, each dog was autopsied to be certain that the biliary system was totally obstructed, and that the catheter was still in place. A liver biopsy was obtained on most of the animals, and cholestasis, portal tract expansion and bile duct proliferation was evident in each case.

Lipoprotein Isolation

The red cells were separated from the plasma by a centrifugation at 2000 rpm for 20 min in a Lourdes LRA refrigerated centrifuge. The low density lipoprotein (LDL S_f 0-20) fraction was prepared from the plasma by a previously described (11) technique. The plasma was made to density 1.006 by additions of Tris 0.03 M, NaCl 0.12 M buffer at pH 7.4, and the very low density lipoproteins (VLDL) removed by an ultracentrifugation at 40,000 rpm for 16 hr. The infranate was again spun at this density to insure the removal of the VLDL. The infranate from repeat centrifugations was adjusted to density 1.063 with 0.145 M NaCl in D_2O and the samples were spun for 24 hr at 40,000 rpm to isolate the LDL. All centrifugations were performed in a Spinco Model L2-50 ultracentrifuge at 8 C, using a 40 rotor.

Analytical Ultracentrifugation

Whole plasma was spun in a Model E ultracentrifuge by a modification of the method of DeLalla and Gofman (12). The samples were made to density 1.063 with 0.145 M NaCl in D_2O and spun in double sectored cells with an appropriate solvent blank using an A and D rotor. All centrifugations were performed at 26 C with a rotor speed at 52,000 rpm. The schlieren displacement was determined, and the flotation velocities calculated from the standard relationship (13).

Polarized Microscopy

A drop of plasma was placed on a microscope slide and covered quickly with a cover slip. The slide was scanned in a polarizing microscope at a magnification of 100. When birefringence was found the sample was viewed at a 400-fold magnification. A quartz first order red compensator was introduced into the field to determined the sign of birefringence.

Electron Microscopy

Whole plasma and isolated LDL were studied by negative staining electron microscopy, both diluted 1- to 20-fold and undiluted. A 200 mesh formvar and carbon-coated grid was used for these studies. The samples were applied for 20 sec and the excess removed by touching to filter paper. A solution of ferritin was then applied and removed after 10 sec. The negative stain, 2% sodium silicotungstate pH 6.9, was applied on the grid for 30 sec and the excess removed. The samples were immediately studied in an AEI electron microscope.

Chemistry

Lipids were extracted from the isolated lipoproteins by the method of Folch et al. (14). Bile acids were extracted from plasma by the procedure of Weiner et al. (15). Biliary lipids were extracted with absolute ethanol using 50 volumes of ethanol per volume of bile. Phospholipids from both bile and plasma were separated by thin layer chromatography using a chloroform-methanol-strong ammonia water-water 50:25:3:1 system on Silica Gel G plates. The phospholipid bands were identified by their comigration with known phospholipid standards. Isolated bands were scraped from the plate and eluted with chloroform-methanol 1:1. The fatty acid compositions were determined for the isolated phospholipids by gas liquid chromatography of their methyl esters. The fatty acids were methylated by the technique of Bragdon and Karmen (16), and the methyl esters were isolated by partitioning into heptane. Fatty acid methyl esters were separated on a 10% EGGSX column at 180 C. The column was standardized with known fatty acid methyl esters. Trihydroxy bile acids were determined by a standard procedure (17), and both the trihydroxy and dihydroxy acids were evaluated by gas liquid chromatography. Methyl and acetyl derivatives were made of the bile acids by a previously described method (18). The derivatized bile acids were run on a QF 1 column at 270 C using 5B cholanic acid as an internal standard. Relative quantities of both bile acids and fatty acids were obtained by triangulation of the gas liquid chromatographic peaks. Phospholipids were obtained by the procedure of Ames and Dubin (19). Choles-

TABLE I

Biliary Lipids after Acute Biliary Obstruction[a]

Time	Total bile acids μg/ml	%	Cholesterol μg/ml	%	Phospholipid μg/ml	%	Total, μg/ml
Prior to obstruction	20,152(2056)[b]	78	129(21)	0.5	5483(358)	21	25,765(2419)
5 days after	9,817(1763)[c]	84	37(5)[c]	0.3	1880(253)[d]	16	11,732(1820)[c]
9 days after	1,676(328)[c]	61	28(3)[c]	1.0	1035(186)[c]	38	2,738(581)[c]
20 days after	1,794(176)[c]	72	19(2)[c]	0.8	687(101)[c]	27	2,500(163)[c]

[a]Values represent means of six obstructed animals.
[b]Standard error of mean is given within parenthesis.
[c]Value is significantly different from preobstructed concentrations at P = .01 level.
[d]Value is significantly different from preobstructed concentrations at P = .05 level.

terols were determined by a modified Abell procedure (20), and proteins by the method of Lowry et al. (21).

Radioactivity was determined in a liquid scintillation system using a 0.5% diphenyl oxazole (DPO) in toluene system for the phospholipid and a 0.6% DPO in dioxane, anisole and dimethoxyethane system for bile acids. Activities were determined in a Packard Model 3375 Liquid scintillation counter with quenching corrected for by an internal standard method.

The low density lipoprotein fraction was delipidated by three 3:1 ethanol ether extractions and two ether washes using 50 volumes of solvent per volume of lipoprotein. The delipidated proteins were reduced with 1% β-mercaptoethanol, dissolved in 1% sodium dodecyl sulfate and run in a 10% polyacrylamide gel (22). The proteins were stained with Coomassie Blue.

Lecithin was prepared from eggs by the procedure of Hanahan et al. (23). This material was found to be pure by thin layer chromatography in both a chloroform-methanol-water 195:75:12 and chloroform-methanol-ammonia-water 50:25:3:1 system. A 1 g quantity of this lecithin was sonicated under nitrogen, using a Branson sonifier, at 4 C in 2 ml of obstructed dog bile, for 20 min. Taurocholate was obtained from Maybridge Research Chemicals (Cornwall, U.K.) and determined to be pure by thin layer chromatography in an isoamyl acetate-propionic acid-N propanol-water 30:30:20:15 system. A 1 g quantity of this taurocholate was dissolved in a 2 ml of obstructed dog bile. Uniformly ^{14}C-labeled lecithin was obtained from New England Nuclear (Boston, Mass.) and 24-^{14}C taurocholate, from Tracerlab (Waltham, Mass.). By the thin layer chromatographic systems indicated above the labeled lipids were greater than 95% pure for lecithin and 80% for taurocholate.

Low Angle X-Ray Scattering

Low angle X-ray studies were performed using an X-ray tube with a copper anode and a Kratky collimation system with automatic step scanner. The entrance slit was 120 μ, the counter slit 200 μ and the distance from sample to counter slit 20.4 cm, which gave a resolution in excess of 800 Å. Scattering curves were registered with a proportional counting tube. Monochromatization to 1.54 Å was achieved

TABLE II

Biliary Bile Acids after Obstruction[a]

Time	% of total Cholic	Chenodeoxycholic	Deoxycholic
Prior to obstruction	69(0.3)[b]	9(0.2)	21(0.4)
5 days after	70(1.7)	16(0.6)[c]	14(1.0)[c]
9 days after	86(1.1)[c]	14(1.1)[c]	[d]
20 days after	86(1.1)[c]	14(1.1)[c]	[d]

[a]Values represent mean per cents by weight of six obstructed animals.
[b]Standard error of mean is given within parenthesis.
[c]Value is significantly different from preobstructed values at at least P = .01 level.
[d]No deoxycholate could be detected at these times.

TABLE III

Plasma Lipids after Acute Biliary Obstruction[a]

Time	μg/ml				
	Phospholipid	Free cholesterol	Cholesteryl ester	Triglyceride	Bile acids[b]
Prior to obstruction	3843(384)[c]	388(17)	1456(182)	530(64)	2.5
6 days after	5262(421)[d]	943(66)[d]	1325(7)	832(220)	
8 days after	7832(940)[d]	1592(119)[d]	1584(24)	560(28)	5.3
14 days after	5863(850)	1634(302)[d]	1585(309)	600(111)	

[a]Values are means of determinations on four acutely obstructed dogs.
[b]Values are means of determinations on two acutely obstructed dogs.
[c]Standard error of the mean is given within parenthesis.
[d]Value is significantly different from preobstructed value at at least P = .05 level.

with a pulse height discriminator and an 8 μ nickel filter. No correction for slit smearing was applied. The liquid sample was kept in a Mark capillary at 22 C. The sample was prepared in 0.03 M Tris, 0.12 M NaCl at pH 7.4. The LDL concentration was 5%. For preparation of the dry sample, after dialysis against H_2O, small portions of the solution were applied to a cellulose acetate foil and allowed to dry. The foil gives no appreciable scattering in the range investigated. For a comprehensive review and further reference on this method see Reference 24.

RESULTS

After ligation of the main bile duct in each of these dogs, the concentrations of each of the major lipids in the biliary system declined (Table I). Within a 3 week period the total biliary lipid levels fell to less than 10% of that in the unobstructed biliary tract, and usually persisted at these low levels for the 6 weeks of observation. In the early stages of obstruction the per cent lipid composition was similar to preobstruction, but at later times phospholipid and free cholesterol comprised somewhat more of the biliary lipid. A change in the relative contents of the biliary bile acids was also observed in these obstructed animals (Table II). As would be anticipated, deoxycholic acid disappeared from the bile shortly after the animal was obstructed.

At the same time the lipid concentrations in the biliary tract were falling, the plasma free cholesterol and phospholipid concentrations increased in each of these dogs (Table III). A modest rise was also observed for the plasma bile acid concentrations after obstruction. The plasma cholesteryl ester concentrations were relatively unchanged. Most of the increment in plasma phospholipid and free cholesterol was isolated in the low density flotation fraction (S_f 0-20) of plasma. The low density phospholipid and free cholesterol concentrations went from 18 and 6 mg/*dl* before obstruction to 255 and 130 mg/*dl* after 9 days of obstruction. No LDL could be detected by analytical ultracentrifugation in normal dog plasma (Fig. 1, top panel). An obvious schlieren peak at S_f12 was identified in the obstructed dogs (Fig. 1, bottom panel). The relative composition of the LDL demonstrated a progressive change during the course of obstruction (Table IV). Relatively less protein and cholesteryl ester and more phospholipid and free cholesterol were seen in this fraction. Whereas prior to obstruction an appreciable fraction of the LDL phospholipid was

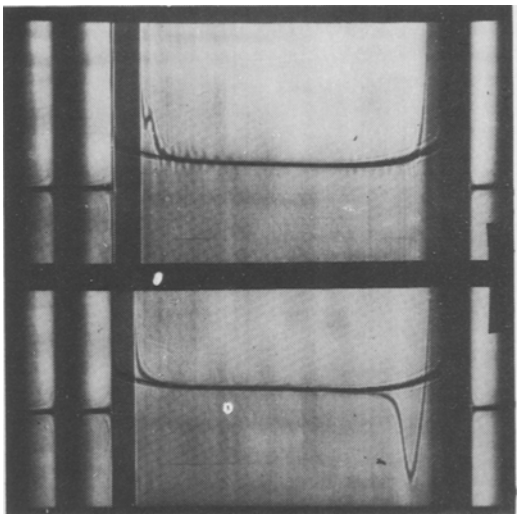

FIG. 1. *Top panel*: Analytical ultracentrifugal pattern of plasma from a dog prior to biliary obstruction. Both pre- and postobstructed samples were run at density 1.063 under conditions described in text, and evaluated for same period of centrifugation. Direction of flotation is from right to left. *Bottom panel*: Analytical ultracentrifugal pattern of plasma from same dog after 5 days of biliary obstruction.

TABLE IV

Low Density Lipoprotein (S_f 0-20) Composition after Acute Biliary Obstruction[a]

Time	Composition, %				
	Protein	Free cholesterol	Cholesteryl ester	Phospholipid	Triglyceride
Prior to obstruction	24	10	32	32 (82)[b]	3
9 days after	10	27	9	48	6
13 days after	7	29	5	51 (99)	8

[a]Values are mean per cents by weight from two acutely obstructed dogs.
[b]Figure in parentheses indicates per cent of phospholipid that was lecithin.

sphingomyelin, i.e., 14%, after obstruction it was almost all lecithin, i.e., 99%. A change in the LDL lecithin fatty acid pattern was also observed after obstruction (Table V). Prior to obstruction LDL lecithin contained only saturated fatty acids. After obstruction oleate and linoleate were now present in the lecithin and relatively less stearate was found. No change was noted in the biliary lecithin fatty acid pattern after obstruction.

Similar to the findings in obstructed humans (5), the lipid isolated in the low density fraction of these obstructed dogs had a lamellar liquid crystalline configuration (Fig. 2A). Indications of a lamellar mesophase structure were obtained both by negative staining electron microscopy and by polarized light microscopy (Fig. 2B) where maltese cross configurations were seen. The signs of birefringence of these structures were noted to be positive, similar to that of mesophase lipid in human biliary obstruction (5). This finding is compatible with the concept that the phospholipid fatty acid chains in these lamellae are normal to the lamellar plane. Low angle X-ray scattering data of the low density fraction at a concentration of 5% lipid revealed no obvious Bragg spacings. The low angle X-ray scattering of the dried low density lipid, however, indicated a pronounced 44 Å spacing (Fig. 2C) similar to the periodicity observed by electron microscopy.

The protein pattern of the plasma LDL fraction changed after biliary obstruction in these dogs (Fig. 3). A new low molecular weight protein of ca. 9000 daltons was isolated in this flotation fraction in the obstructed dogs. Since the protein pattern of normal canine LDL has not been established, it might be argued that this is an impurity of the preparation. However no such protein was ever found in the low density fraction of these dogs prior to obstruction.

After 2 weeks of obstruction the biliary tract of these dogs contained relatively little lipid, and plasma concentrations of phospholipid and cholesterol were either stable or decreased slowly. At this time a dispersion of pure egg lecithin was placed in the biliary tree, being careful not to increase the pressure of this system. Shortly after the introduction of a sonicated 1 g lecithin dispersion into the chronically obstructed biliary tract of two dogs, an increment was noted in the plasma lecithin concentrations of both (Table VI). No appre-

TABLE V

Lecithin Fatty Acid Composition after Acute Biliary Construction[a]

Time	Composition, %			
	16:0	18:0	18:1	18:2
	(Low density (S_f 0-20) lecithin			
Prior to obstruction	40.6	59.5	b	b
13 days after	37.0	43.6	12.8	6.6
	Biliary lecithin			
Prior to obstruction	33.5	21.5	24.0	21.0
13 days after	31.0	24.5	25.0	20.0

[a]Values are means of determinations on two dogs and expressed as per cent composition by weight.
[b]No oleate or linoleate could be detected in LDL lecithin in either of preobstructed animals.

ciable change in plasma lipid was seen during this time interval for the control animal. Assuming a plasma volume of 50 ml/kg (25), the lecithin increment of ca. 1.8 mg/ml observed after 1 g of lecithin was placed in the biliary tract suggests that nearly all of it accumulated in the plasma of these 10 kg dogs. As in acute obstruction the biliary lecithin concentrations decreased as the plasma level increased (Table VI). An increase in the plasma free cholesterol concentration accompanied the plasma lecithin increment. The cholesteryl ester concentrations remained relatively constant. These plasma lipid alterations seen after increasing the biliary lecithin concentration in a chronically obstructed system closely simulated the lecithin and free cholesterol increases observed after acute obstruction.

The effect of an isolated increase in biliary taurocholate concentration on plasma lipids was also evaluated in these chronically obstructed dogs. A 1 g dispersion of sodium taurocholate was introduced into the lipid-depleted biliary system of another pair of dogs, which had been obstructed for 2 weeks. A modest rise in plasma bile acid was noted (Table VI), but in contrast to the lecithin studies a mean decrease of ca. 80 mg/*dl* in plasma phospholipid and ca. 60 mg/*dl* in total cholesterol concentrations was noted. Cholesteryl ester concentrations decreased earlier and to a greater extent than free sterol after increasing biliary taurocholate levels.

Studies were performed introducing uniformly labeled ^{14}C-lecithin and 24-^{14}C-taurocholate into the bile ducts of the two dogs obstructed for 2 weeks. The plasma recovery of the labeled lipids were determined for the following week. The maximum activity of taurocholate occurred 8 hr later and assuming a plasma volume in the dog of 50 ml/kg (25), less than 3% of the injected taurocholate activity was recovered in the plasma of the obstructed dogs at this time. About 40% of the biliary lecithin activity was recovered as radioactive lecithin in the plasma of one of the dogs at 2 days (Fig. 4). Radioactivity was not observed in other plasma complex lipids up to 3 days.

DISCUSSION

The plasma lipid alterations that result after biliary obstruction are somewhat similar for both canines and humans. It is predominantly the lecithin and free cholesterol concentrations that increase in both species. The plasma lipid increment is isolated mainly in the low density (S_f 0-20) flotation fraction in each of the species. The structure of the plasma low density lipoproteins in human biliary obstruction has

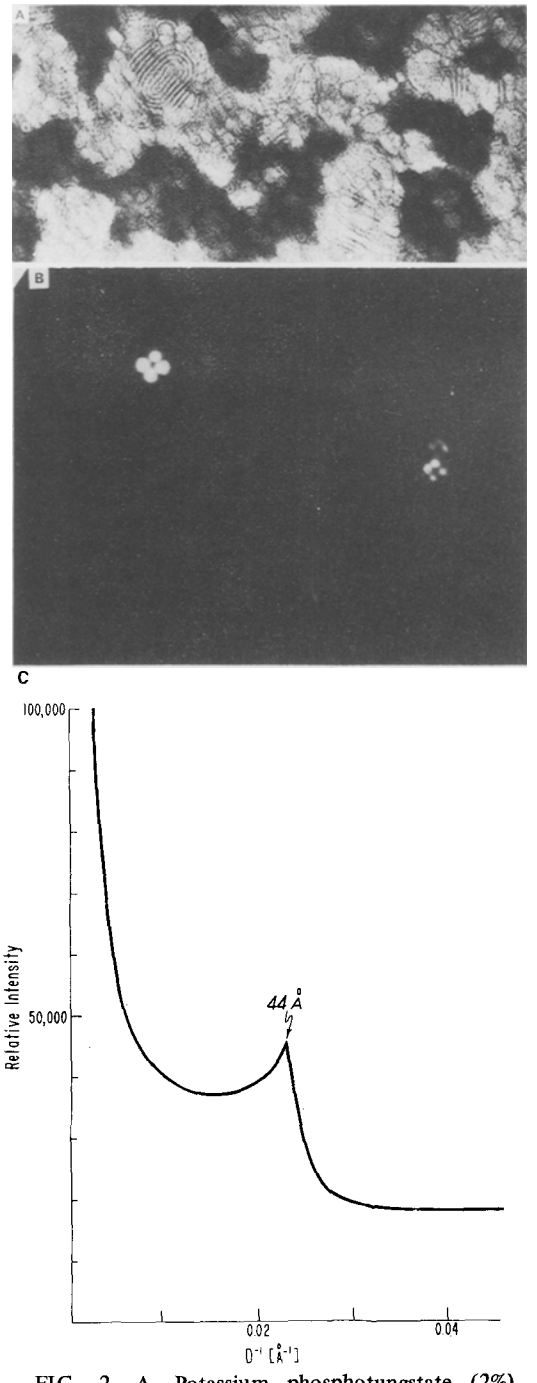

FIG. 2. A. Potassium phosphotungstate (2%) stained low density lipoprotein fraction from dog with biliary obstruction. Ferritin markers are included (70 Å ring shapes) to determine size. Magnifications at 400,000 X. B. Polarized microscopic observation of isolated low density lipoproteins from a biliary obstructed dog x 1200. C. Low angle X-ray scattering pattern of dried low density lipoproteins from an obstructed dog performed as described in the text.

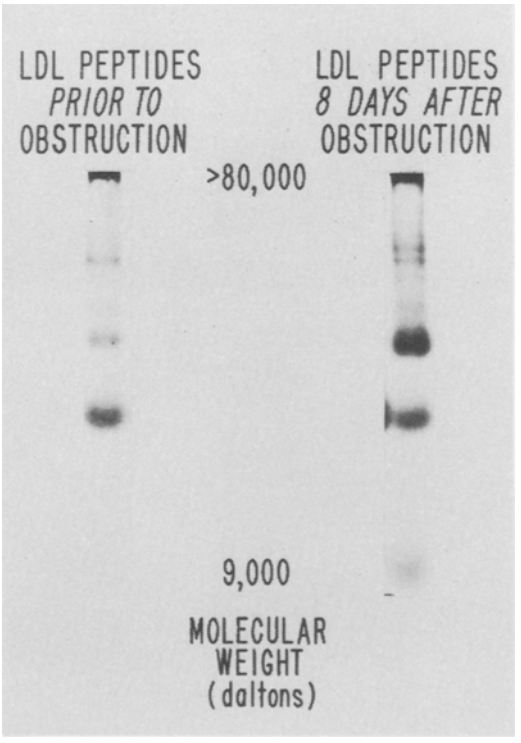

FIG. 3. Normal and obstructed low density apoproteins evaluated by sodium dodecyl sulfate-polyacrylamide electrophoresis on a 10% polyacrylamide system. Molecular weight range is indicated.

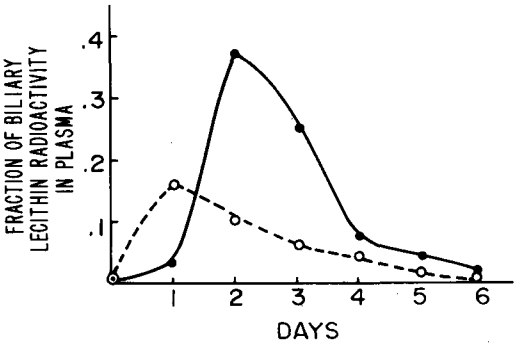

FIG. 4. Fraction of biliary lecithin radioactivity recovered in plasma as a function of time after instillation of 2 μc of uniformly labeled lecithin into biliary tract. The study of dog 11 is indicated by_____ and dog 14 by ------. Both dogs were assumed to have plasma volumes of 50 ml/kg body wt.

been noted to differ markedly from the normal spherical human low density lipoprotein (4,5). Configurations with the appearance of flattened vesicles, stacked lamellae and lamellar whorls were found by negative staining electron microscopy. The lamellar mesophase nature of the human obstructed lipoprotein has also been documented by polarized microscopy and low angle X-ray scattering. A lamellar liquid crystal structure was detected similarly in the plasma of these obstructed dogs by both polarized and electron microscopy. The bulk of this mesophase appeared to be very finely dispersed with diameters of the lamellar spherules varying from 150 to 400 Å. The fine nature of this dispersion may explain the inability to discern distinct Bragg spacing by low angle X-ray techniques for the material, despite clear indication of a smectic mesophase by both polarized and electron microscopy. Bragg spacings have been noted to disappear in liquid crystal dispersions after prolonged sonication had reduced the size of the dispersion (26).

Although the new lipoprotein species appearing in the canine LDL fraction after biliary obstruction could not be isolated from the normal lipoproteins, the change in total LDL composition provides a suggestion as to the composition of this abnormal lipid transport form. Lecithin and free cholesterol in approximately a 1:1 molar ratio were the main constituents of this LDL increment. This appears analogous to human obstruction where lecithin and cholesterol in a 1:1 molar ratio were the predominant components of the bilayer structures (5). Also similar to the human postobstructive state is the appearance of a new low molecular weight protein in the LDL fraction.

The simultaneous decrease in biliary tract lipid with increasing plasma lipid concentrations suggests a reflux of biliary lipid to plasma after acute obstruction. The recovery of a large proportion of both labeled and unlabeled biliary lecithin in the plasma of these obstructed dogs strongly supports net flux of biliary lecithin to plasma. The shift of the plasma phospholipid pattern toward lecithin with a fatty acid composition more like biliary lecithin also supports the reflux of biliary lecithin to plasma. The entire increment in plasma phospholipid, which was observed after acute biliary obstruction, could be the result of only 4 days of normal biliary phospholipid output that had refluxed into plasma. Although there is some net reflux of bile acid from the biliary tree to the plasma as a consequence of obstruction, the bile acids do not partition in the plasma to nearly the extent of lecithin.

The decline in biliary cholesterol concentration at a time when the plasma levels increase also suggests a net transfer of biliary cholesterol to plasma. The magnitude of this plasma cholesterol increment, following acute obstruction, obviously cannot be accounted for by biliary cholesterol reflux alone. Assuming a

TABLE VI

Effect of Increased Biliary Lecithin and Taurocholate Concentrations on Plasma Lipids of a Chronically Obstructed Dog[a]

Experiment	Time, days	μg/ml					
		Cholesterol				Phospholipid	
		Free		Ester			
		Dog 4	Dog 6	Dog 4	Dog 6	Dog 4	Dog 6
Control	0	968	835	1442	1265	3478	3262
	1	908	742	1225	1053	3124	3244
	4	788	736	1100	1007	3791	3770
	6	936	822	1379	1153	3614	3546
Lecithin[b]		Dog 8	Dog 12	Dog 8	Dog 12	Dog 8	Dog 12
	0	987	1203	990	1110	3785	3975
	1	1108	1752	965	995	4432	4478
	4	1422	1770	1148	1252	5630	5740
	6	1612	1888	1143	1217	5654	5746
Taurocholate[c]		Dog 9	Dog 10	Dog 9	Dog 10	Dog 9	Dog 10
	0	780	326	1236	1806	3205	3251
	1	764	342	1007	1543	2873	3127
	2	702	286	1026	1386	2859	2853
	4	612	266	857	1235	2418	2430

[a]Day "O" value was obtained on 17th postobstructed day just prior to placing 1 g amounts of lecithin and taurocholate in biliary tracts of respective experimental groups.

[b]Biliary lecithin concentrations were 18.6 mg/ml 4 hr after lecithin installation and 2.2 mg/ml 4 days later.

[c]Plasma bile acids went from 2.8 μg/ml before to 8.2 μg/ml 2 days after taurocholate.

normal biliary cholesterol production and complete reflux into plasma, a maximum of only 15% of the plasma cholesterol increment could be derived from this source. The substantial increase in plasma cholesterol, noted when only lecithin refluxed from the cholesterol-depleted biliary tract, also indicates that this plasma cholesterol increment is from a source other than the biliary tree. Byers and Friedman (27) have very carefully documented the origin of the plasma cholesterol rise, which they saw after a plasma infusion of egg lecithin in the rat. Their data suggest that an appreciable amount of the phosphate-induced hypercholesterolemia was derived from hepatic cholesterol, both newly synthetized and preformed. The skin was thought to be another source of this plasma cholesterol. Interestingly, when a plasma lecithin infusion was continued for 24 hr a substantial stimulation of cholesterol synthesis was observed in both the liver and intestine (28). The increment in hepatic cholesterol synthesis from this lecithin infusion was about the same as that noted in an obstructed rat (29). The mechanism for the accelerated cholesterol synthesis in biliary obstruction may be due in part to the secondary effect of biliary lecithin reflux into plasma with its known capacity to enhance cholesterogenesis.

The fall seen in both plasma phospholipid and cholesterol concentrations when the taurocholate concentrations were increased in the chronically obstructed biliary tree is unexplained. Investigations are currently proceeding into the mechanism of this phenomenon.

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REFERENCES

1. Fredrickson, D.S., A.V. Loud and B.T. Hinkelman, J. Exp. Med. 99:43 (1954).
2. Russ, E.M., J. Raymunt and D.P. Barr, J. Clin. Invest. 35:133 (1956).
3. Ahrens, E.H., Jr., and H.G. Kunkel, Ibid. 28:1565 (1949).
4. Hamilton, R.L., R.J. Havel and J.P. Kane, Science 172:475 (1971).
5. Quarfordt, S.H., H. Oelschlaeger and W.R. Krigbaum, J. Clin. Invest. 51:1979 (1972).
6. Howell, J.I., J.A. Lucy, and R.C. Pirola, Biochim. Biophys. Acta 210:1 (1970).
7. Forte, T., K.R. Norum and J.A. Glomset, J. Clin. Invest. 50:1141, (1971).
8. Friedman, M., and S. Byers, Amer. J. Physiol. 188:337 (1957).

9. Byers, S.O., and M. Friedman, Ibid. 168:138 (1952).
10. Makino, I., S. Nakagawa and K. Mashimo, Gastroenterology 56:1033 (1969).
11. Havel, R.J., H.A. Eder and J.H. Bragdon, J. Clin. Invest. 34:1345 (1955).
12. Delalla, O., and J.W. Gofman, "Methods of Biochemical Analysis," Edited by D. Glich, Interscience Publishers, New York, 1954, p. 1.
13. Shackman, H.K., "Ultracentrifuge in Biochemistry," Third edition, Academic Press, New York, 1959, p. 10.
14. Folch, J., M. Lees and G.H. Sloane-Stanley, J. Biol. Chem. 226:497 (1957).
15. Weiner, I.M., J.E. Glasser and L. Lack, Amer. J. Physiol. 207:964 (1964).
16. Bragdon, J.H., and A.K. Karmen, J. Lipid Res. 2:400 (1961).
17. Irwin, J.L., C.G. Johnston and J. Kopala, J. Biol. Chem. 153:439 (1944).
18. Sandberg, D.H., J. Sjövall and K. Sjövall, J. Lipid Res. 6:182 (1965).
19. Ames, B.N., and D.T. Dubin, J. Biol. Chem. 235:769 (1960).
20. Abell, L.L., B.B. Levy and B.B. Brodie, Ibid. 195:357 (1952).
21. Lowry, O.H., N.J. Rosebrough and A.L. Farr, Ibid. 193:265 (1951).
22. Weber, K., and M. Osborn, Ibid. 244:4406 (1969).
23. Hanahan, D.J., M.B. Turner and M.E. Jayko, Ibid. 192:623 (1951).
24. Alexander, L.E., "X-Ray Diffraction Methods in Polymer Science," Wiley, New York, 1969.
25. Reihart, O.F., and H.W. Reihart, "Canine Medicine," First edition, Edited by E.J. Catcott, Illinois. American Veterinary Publications Inc., Illinois, 1968, p. 341.
26. Wilkins, M.H., A.E. Blaurock and D.M. Engelman, Nature (New Biol.) 230:72 (1971).
27. Byers, S.O., and M. Friedman, Lipids 4:123 (1969).
28. Byers, S.O., M. Friedman and T. Sugiyama, J. Biol. Chem. 237:3375 (1962).
29. Weis, H.J., and J.M. Dietschy, J. Clin. Invest. 48:2398 (1969).

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SHORT COMMUNICATIONS

Bis-(Monoacylglyceryl)-Phosphate of Rat and Human Liver: Fatty Acid Composition and NMR Spectroscopy

ABSTRACT

Bis-(monoacylglyceryl)-phosphate was isolated from liver lysosomes that had been prepared from rats pretreated with Triton WR-1339. Analysis of the fatty acids in this lipid by gas chromatography and mass spectrometry revealed that they were composed of 69% docosahexenoic acid. NMR spectroscopy of bis-(monoacylglyceryl)-phosphate isolated from liver of a patient who had a lipid storage disease revealed that the peak corresponding to the chemical shift of protons of esterified secondary carbons of glycerol was absent. This suggests that fatty acids are esterified to the primary position of both glycerols.

An unusual phospholipid, bis-(monoacylglyceryl)-phosphate, was first identified in mammalian tissues by Body and Gray (1). Details of the structure of this lipid were determined from samples obtained from lung (1), human pathological tissues (2,3), the liver of humans and rats treated with the drug 4,4-diethylaminoethoxy hexestrol (4) and rat liver lysosomes pepared following loading with the detergent, Triton WR-1339 ("tritosomes") (5). These studies have demonstrated a composition consisting of two fatty acids and two glycerol moieties per phosphorus and have established that each of the fatty acids is esterified to a different glycerol. The position of esterification of the fatty acids to each of the glycerols has not been determined. Body and Gray (6) and Seng et al. (3) have pointed out the high proportion of unsaturated fatty acid in the lipid and have suggested, by analogy with other phospholipid classes where more structural data is known, that this indicates esterification of fatty acids in the secondary positions.

We have examined the fatty acid composition of samples of bis-(monoacylglyceryl)-phosphate from both human and rat sources and have not only confirmed that the lipid contains mainly unsaturated fatty acid, but have also found that it may be unsaturated to an unusual degree. We also report the results of NMR spectroscopy carried out on a sample of human origin, which are compatible with a structure in which both fatty acids are esterified to the primary position of the glycerol moieties.

Bis-(monoacylglyceryl)-phosphate was isolated from rat liver "tritosomes" and was identified by procedures described earlier (5). Solvents used for isolations contained 2,6-di-*t*-butyl-*p*-cresol at a concentration of 0.005%. The fatty acid composition was determined by gas chromatography of the methyl esters, which were obtained as described previously (5). Analysis was carried out on an F and M 402 gas chromatograph fitted with columns of 6% diethyleneglycol succinate and 3% SE-30. Yield of fatty acid methyl ester was determined to be 96% or higher, both by colorimetric estimation of ester (7) and weighing the peaks on the chromatograms and comparing the sum to the weight of the peak corresponding to a known quantity of added internal standard of non-

TABLE I

Fatty Acid Composition of Bis-(Monoacylglyceryl)-Phosphate

Identification	Per cent		
	Rat liver tritosomes		Human liver
		(Hydrogenated)	
C14:0	<1	1	<1
C16:0	3	6	6
C16:1	1		2
Unidentified			3
C18:0	1	13	5
C18:1	5		57
C18:2	6		10
C20:0		7	
C20:1			2
C22:0	4	73	3
C20:5	2		
C24:0	2		
C24:1	2		3
C22:5	4		
C22:6	69		9

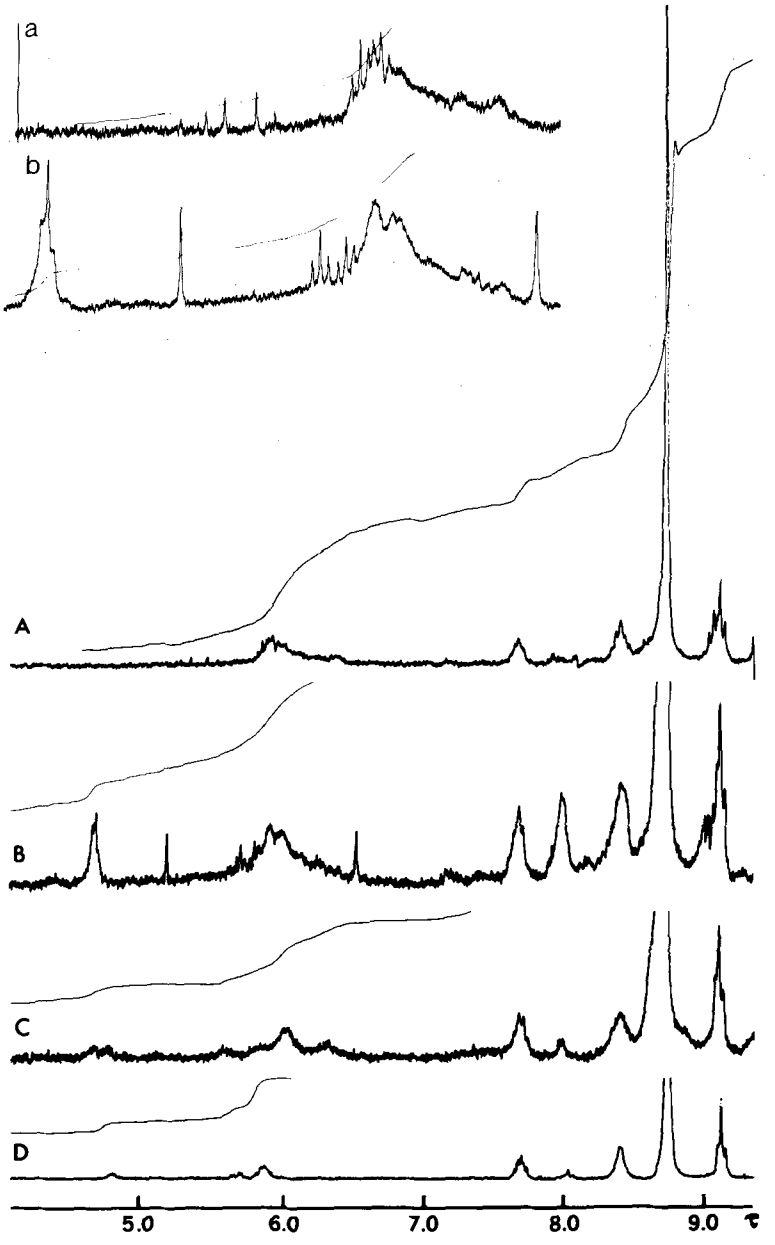

FIG. 1. NMR spectra of human liver bis(monoacylglyceryl) phosphate after (A) and before (B) hydrogenation, phosphatidylglycerol (C) and tetrapalmitoyl bis-(L-α-glyceryl) phosphoric acid (D). Inserts a and b are magnifications of the portions between 4.6 and 6.3τ of spectra A and B, respectively.

adecanoic methyl ester. Peaks were identified by comparison with known standards and by plotting relative log retention times obtained on both types of column. To confirm identification of unsaturated peaks, samples of fatty acid methyl ester were also run after catalytic hydrogenation (8). The fatty acid composition determined is given in Table I. The identity of the peak having the retention time of 22:6 was confirmed by mass spectrometry. The following fragments that support a structure of 22:6 ω3 were found: 342, M^+; 327, M-CH_3 terminal; 313, M-C_2H_5 terminal; 311, M-OCH_3; 299, M-C_3H_7 terminal; 273, M-C_5H_9 terminal, indicating a double bond at C_{19} and 206, C_1-C_{12} fragment.

These results show that up to 89% of the fatty acids in bis-(monoacylglyceryl)-phosphate may be unsaturated, with 70% being 22:6. It is of note that the sample so highly enriched in 22:6 was isolated from rat liver "tritosomes," which have the characteristics of secondary lysosomes (9). The possible contribution of this highly unsaturated phospholipid to the specific biological roles of the vacuolar apparatus is therefore of some interest.

A sufficient amount of sample of bis(monoacylglyceryl) phosphate was isolated from the liver of a patient who died with an undefined form of lipid storage disease (10) to allow NMR spectroscopy. The lipid accounted for 5.3% of total phospholipid (P) and was identified as described for the rat lipid. Its fatty acid composition is shown in Table I. NMR spectra were recorded from 6-10 mg of lipid dissolved in chloroform using a Varian Associates HR-220 spectrometer with tetramethylsilane as standard. Spectra obtained for bis-(monoacylglyceryl)-phosphate and two standard lipids for comparison, synthetic tetrapalmitoyl bis-(L-α-glyceryl)-phosphoric acid and phosphatidyl glycerol (Supelco, State College, Pa.), are shown in Figure 1.

The spectra reveal peaks at 9.1, 8.7, 8.0, 7.7τ, between 5.7 and 6.3 and between 4.6 and 4.8τ, which are accounted for by terminal methyl protons, methylene protons, methylene protons adjacent to double bonds, methylene protons adjacent to carboxyl groups, glycerol protons except protons of the secondary carbon of glycerol when esterified and ethylenic protons, respectively (11,12,13). All three substances reveal peaks at 4.6-4.8τ in keeping with the presence of unsaturated fatty acids present in both bis-(monoacylglyceryl)-phosphate and phosphatidylglycerol and esterified fatty acids in the second position present in tetrapalmitylbis (L-α-glyceryl)-phosphoric acid and phosphatidylglycerol.

The sample of bis-(monoacylglyceryl)-phosphate was then hydrogenated (8) twice. This was shown to be complete by gas chromatography of fatty acid methyl esters as described above. The NMR spectrum (Fig. 1A and insert a) revealed now that all peaks at 4.6-4.8τ had disappeared. The areas under the various peaks do not in most instances adhere to stoichimoetry expected for the various proton positions. For example, if it is assumed that both fatty acids in bis-(monoacylglyceryl)-phosphate are esterified in the primary position so that none of the glycerol protons will have a chemical shift in the 4.6-4.8τ region, and the contribution of the ethylenic protons is calculated from Table I, the theoretical ratio of peaks 4.6-4.8:5.7-6.3 is 5.1:10. The value determined from Figure 1 is about half the theoretical value. However a small error in the determination of C22:6 by gas chromatography will be amplified in the calculation of the above ratio. Therefore, even if we assume that half of the area of the peaks at 5.7-6.3 are accounted for by contaminant and that only one of the two fatty acids resides in the secondary position, a peak should be visible in the hydrogenated sample at 4.6-4.8τ. Because such a peak could not be detected, we conclude that both fatty acids are esterified to the primary position.

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REFERENCES

1. Body, D.R., and G.M. Gray, Chem. Phys. Lipids 1:254 (1967).
2. Rouser, G., G. Kritchevsky, A. Yamamoto, A.G. Knutson and G. Simon, Lipids 3:287 (1968).
3. Seng, P., H. Debuch, B. Witter and H.R. Wiedemann, Hoppe Seyler's Z. Physiol. Chem. 352:280 (1971).
4. Yamamoto, A., S. Adachi, T. Kitani, Y. Shinji, K. Seki, T. Nasu, and M. Nishikawa, J. Biochem. (Tokyo) 69:613 (1971).
5. Wherrett, J.R., and S. Huterer, J. Biol. Chem. 247:4114 (1972).
6. Body, D.R., and G.M. Gray, Chem. Phys. Lipids 1:424 (1967).
7. Bradley, R.M., Biochim. Biophys. Acta 106:417 (1965).
8. Eybel, C.E., and R.G. Simon, Lipids 5:590 (1970).
9. Wattiaux, R., M. Wibo, and P. Baudhuin, in "Ciba Foundation Symposium on Lysosomes," Edited by A.V.S. de Reuck and M.P. Cameron, Little Brown, Boston, 1963, p. 176.
10. Wherrett, J.R., and N.B. Rewcastle, Clin. Res. 17:665 (1969).
11. Hopkins, C.Y., in "Progress in the Chemistry of Fats and Other Lipids," Vol. 8, Part 2, Edited by R.T. Holman, Pergamon Press, Oxford, 1965, p. 215.
12. Chapman, D., "The Structure of Lipids," Wiley and Sons, New York, 1965, p. 194.
13. Olsen, R.W., and C.E. Ballou, J. Biol. Chem. 246:3305 (1971).

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Volume-Dependent Transfer of Free Fatty Acids from Ehrlich Ascites Carcinoma to Host Tissues

ABSTRACT

The recovery of [9,10-^{3}H] palmitate complexed to mouse serum albumin was studied 5 sec after ip injection into mice bearing Ehrlich ascites carcinomas. Only about 55% of the injected radioactivity remained in the tumor cells, tumor extracellular fluid and peritoneal washings in mice having ca. 5 ml of the ascites tumor (cells plus extracellular fluid). However the recoveries were nearly quantitative from tumors of larger volume ($\geq$ 8 ml). A model is proposed to account for the volume-dependent transfer of palmitate from tumor to host in these cancerous mice.

During a recent study of free fatty acid (FFA) metabolism in mice bearing Ehrlich ascites carcinomas, we observed low "zero-time" recoveries of ip-injected [9,10-^{3}H] palmitate from the peritoneal cavity of animals having 5-7 ml of tumor (cells plus extracellular fluid; unpublished observations). On the other hand, in experiments of longer duration but using mice with larger tumors, recoveries were considerably higher, even though some of the injected fatty acid had been metabolized. The apparent rapid loss of tracer from the tumor fluid of some mice—within 5 sec after ip injection—contrasted markedly with the slow transport of FFA from the ascitic fluid to the host tissues after that time. This suggests that, during the time required for the tracer to equilibrate with the endogenous FFA pool of the ascitic fluid, the tracer and endogenous FFA pools were handled differently, depending upon the volume of tumor. Since a volume-dependent transport of the tracer could influence seriously the interpretation of kinetic studies, we have carried out a study of FFA "transport" during the first 5 sec after ip injection of [9,10-^{3}H]-palmitate into mice bearing Ehrlich ascites carcinomas of varying volumes. Our data establish that there is indeed a rapid, volume-dependent transfer of tracer from the ascitic fluid to the host and that, under the present conditions, this transfer may be reduced to negligible proportions by using tumors of large volume.

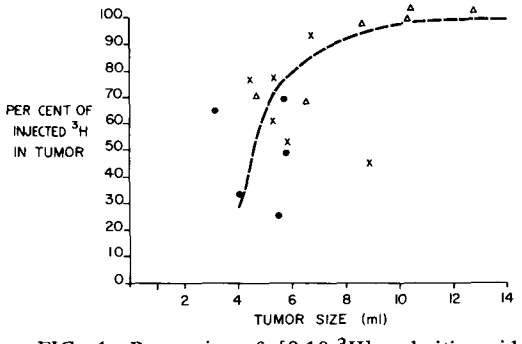


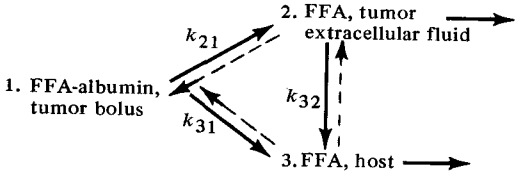
FIG. 1. Recoveries of [9,10-^{3}H] palmitic acid from the peritoneal cavities of mice bearing Ehrlich ascites carcinoma, 5 sec after ip injection of the tracer. The labeled fatty acid was injected as a complex (mouse serum). The tumor volumes were varied by using tumor harvested at the following times after inoculation of 1.2 x 10^7 cells: 5 days (•), 7 days (X) and 9 days (△). The curve was drawn freehand, without assuming any particular mathematical function.

Ehrlich ascites carcinomas (originally obtained from R.W. McKee, Dept. of Biological Chemistry, UCLA School of Medicine) were grown in male, Swiss-Webster mice (Hilltop Lab Animals, Inc., Los Angeles). [9,10-^{3}H] palmitic acid, 500 mCi/mM, was obtained from Amersham-Searle, Inc. and was purified by extraction from hexane into a sodium carbonate solution, followed by acidification with H_2SO_4 and reextraction with hexane. Radiochemical purity was evaluated by thin layer chromatography (1) using a solvent system of *n*-hexane-ethyl ether-acetic acid 166:32:2. At least 98% of the radioactivity migrated as FFA. Tumor-bearing mice were injected ip with 100 μl of the labeled palmitate complexed (2) to mouse serum albumin as follows: The labeled palmitic acid was dissolved in a slight excess of KOH in methanol, the solution taken to dryness, then, after addition of 0.85% NaCl, heated to 60-70 C until total solubilization of the palmitate; an eight-fold volume of mouse serum was then added to the warm solution. The final molecular ratio of added (radioactive) palmitate-serum albumin was ca. 1, and the ratio of total (added and already present) FFA-albumin was ca. 2. Tumor volumes during tracer experiments were varied by using 35-40 g mice injected at varying times (5-12 days) after ip inoculation with 1.2 x 10^7 washed tumor cells. The mice were killed by cervical fracture 5 sec after injection of the labeled palmitate. During the 5 sec period, the abdomens were massaged gently and the mice were rotated from side to side to enhance mixing of the tracer within the ascitic fluid. The skin was removed rapidly, an incision was

made in the peritoneum, and tumor was collected by free drainage. The peritoneal cavity was washed with 10 ml saline and the washings collected separately. Volumes of total tumor cells plus extracellular fluid, and of packed cells (after centrifugation) in both the original tumor collection and in the washings, were recorded. Aliquots of the initial tumor were taken, diluted 10-fold with saline, and counted directly by liquid scintillation spectrometry. Aliquots of the washings were also assayed for ^3H, but without further dilution. Appropriate corrections for quenching were made. The sum of the radioactivity in the entire peritoneal cavity (original tumor and washings) was then calculated for each mouse.

The percentage of injected [9,10-^3H] palmitate found in the tumor cells and extracellular fluid 5 sec after ip injection of tracer is plotted as a function of total tumor volume in Figure 1. The ^3H recoveries were low in every case in which tumors of less than 6 ml volume were used, and, with one exception, the recoveries were quantitative in tumors of 8 ml or more. The loss of ca. 50% of the injected dose in 5 sec in the case of one group of mice (Fig.1, closed circles, average) may be compared with the fractional rate of disappearance of ^3H-labeled palmitate from the ascitic fluid shortly after that time; namely, 50% in 120-210 seconds (3; and unpublished observations). We can exclude the possibility that tumor cells are competing in an important way with receptor sites lining the peritoneal cavity during the first 5 sec, since only ca. 10% of the radioactivity in the peritoneal cavity was associated with the tumor cell lipids, even after 30 sec, under conditions (tumor ca. 6 ml) in which ca. 30% of the injected dose was lost within 5 sec (unpublished observations).

As a basis for the interpretation of these data, we have hypothesized the following simplified model in which the artificial FFA-albumin complex is transferred to the host either from the injected bolus (via k_{31}) or after the tracer FFA has transferred to a more physiological FFA-protein complex in the tumor extracellular fluid (via k_{21} and k_{32}).



Our data indicate that the larger the volume of tumor, the less the chances are for the tracer in the bolus to make contact with the host receptors that line ther peritoneal cavity or the tissues therein, i.e., the larger the tumor volume, the greater the chances for FFA transport via k_{21} to be "completed" without loss of tracer via k_{31}. The transfer of physiological FFA from tumor to host (via k_{32}) is known to be slow (unpublished observations). Further studies are required in order to establish whether the extremely rapid transfer via k_{31} that we have studied here reflects the use of a nonphysiological FFA-albumin complex (4) or whether the bolus loss in 5 sec is that that one expects from low concentrations of a truly physiological FFA-albumin complex prior to mixing with the larger extracellular FFA pool in the ascitic fluid.

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REFERENCES

1. Baker, N., and M.C. Schotz, J. Lipid Res. 5:188 (1964).
2. Friedberg, S.J., W.R. Harlan, Jr., D.L. Trout and E.H. Estes, Jr., J. Clin. Invest. 39:215 (1960).
3. Spector, A.A., Cancer Res. 27:1580 (1967).
4. Kessler, J.I., M. Demeny, and H. Sobotka, J. Lipid Res. 8:185 (1967).

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Methodology for Separation of Gangliosides from Potential Water-Soluble Precursors

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ABSTRACT

The following methods were examined for their ability to remove labeled nucleotide sugars and other water-soluble precursors from ganglioside preparations: enzymatic digestion of nucleotide sugars followed by dialysis; silicic acid and Sephadex G-25 column chromatography; ascending paper chromatography; high voltage paper electrophoresis; thin layer chromatography; co-precipitation of gangliosides and bovine serum albumin with trichloroacetic acid. A procedure was developed which allows these complex glycosphingolipids to remain in the chloroform-rich lower phase during conventional Folch partitioning.

INTRODUCTION

In vitro studies of the individual reactions responsible for the biosynthesis of gangliosides usually employ specific radioactive nucleotide sugar donors (UDP-glucose, UDP-galactose, UDP-N-acetylgalactosamine or CMP-sialic acid). A variety of methods and approaches have been devised to accomplish the separation of remaining labeled precursors from the reaction product for reliable assay.

Mixtures containing labeled gangliosides produced both in vitro and in vivo and labeled water-soluble precursors have been treated with snake venom phosphodiesterase and bacterial alkaline phosphatase followed by dialysis to eliminate the nucleotide sugar contaminants (1). This method may lead to losses if the ganglioside critical micelle concentration is not exceeded (2,3) and is a bit tedious for routine purposes.

Complete separation of gangliosides from nucleotide sugars was reported, employing ascending paper chromatographic (4) and high voltage paper electrophoretic procedures (4,5) with sodium tetraborate (pH 9.0) as the developing solvent or buffer. Fractionation of crude lipid extracts was reported utilizing Sephadex G-25 for removal of a variety of water soluble contaminants, including amino acids, carbohydrates, nucleotides, and inorganic phosphate (6). A Silica Gel G column chromatographic procedure has been used for the removal of contaminating lipids from organic extracts containing gangliosides (7).

This paper reports experiences in this laboratory with available techniques for removal of water-soluble contaminants from ganglioside mixtures. A procedure exploiting early observations on gangliosides (8-10) has been developed based upon the partitioning of gangliosides into a chloroform-rich phase to separate them from nucleotide sugars and other water-soluble contaminants.

MATERIALS

Mixtures of bovine brain gangliosides, purified G_{M1} and G_{M2}, (Nomenclature of Svennerholm, [11]) were isolated by conventional techniques (12,13). Crude lipid extracts (2:1 $CHCl_3$-MeOH) from bovine brain were used without further purification.

Nucleotide sugars, UDP-glucose (sodium salt) and UDP-galactose (potassium salt) were purchased from P.L. Biochemical, Inc. (Milwaukee, Wis.). Crystalline bovine serum albumin (Fraction V), synthetic N-acetylneuraminic acid (Type IV) and *Crotalus adamanteus* venom phosphodiesterase (SA 0.64 units/mg) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Worthington Biochemical Corp. (Freehold, N.J.) was the commercial source of bacterial alkaline phosphatase (SA 34.5 units/mg).

[^{3}H-]G_{M1} (SA 2.0 x 10^6 cpm/μmol) was prepared by the galactose oxidase-sodium [^{3}H]-borohydride method (14), which results in the introduction of ^{3}H specifically at carbon atom 6 of the terminal galactose residue. [^{3}H-] N-acetylgalactosamine (SA 1.19 x 10^8 cpm/μmol) was prepared by the acetic anhydride method (15). New England Nuclear (Boston, Mass.) was the commercial source of [^{3}H]-1-D glucose (SA 6.93 x 10^8 dpm/μmol), UDP-[^{14}C]-glucose (SA 4.4 x 10^8 cpm/μmol) and UDP-[^{14}C]-galactose (SA 4.2 x 10^8 cpm/μmol).

Silicic acid (SilicAR, special CC-7) was purchased from Mallinckrodt Chemical Works (St. Louis, Mo.). Sephadex G-25 (fine) was obtained

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from Pharmacia Fine Chemicals, Inc. (Piscataway, N.J.). Reeve Angel (Clifton, N.J.) was the commercial source of Whatman 3 MM chromatographic paper (0.33 mm). Precoated 250 micron Silica Gel G (TLC) plates (20 x 20 cm) were purchased from Analtech, Inc. (Newark Del.). Cellulose dialysis membrane (5/8 in, size 18) was obtained from VWR Scientific (Boston, Mass.). All solvents were reagent grade and used without further purification.

METHODS AND RESULTS

A. Enzyme Treatment and Dialysis

In an attempt to reproduce a general in vitro incubation condition, 200 μl of a suspension containing 1 mg lyophilized bovine white matter (400 μg protein), in a solution containing 0.90% NaCl, 1.15% KCl, 2.11% KH_2PO_4, 1.85% $MgSO_4$, 1.3% $NaHCO_3$ and buffered with 0.1M Na_2HPO_4-NaH_2PO_4 (pH 7.4) was employed. This solution will be referred to as *incubation mixture I*.

Immediately after the addition of carrier bovine brain ganglioside (5 μmol) and either labeled nucleotide sugar (0.027-0.131 nmol) or [^{3}H]-G_{M1} (5-11 nmol), to the incubation media, 3 ml of $CHCl_3$-MeOH (2:1) was added. The contents of individual tubes were evaporated to dryness, employing N_2 at a temperature below 40 C. Enzyme digestion (1) was accomplished by incubation with 10 μg bacterial alkaline phosphatase, 10 μg snake venom phosphodiesterase, 0.3 ml 1.0 M Tris-HCl buffer (pH 8.5), 0.3 ml 0.3 M magnesium acetate and 2.4 ml distilled H_2O for 2 hr at 37 C. Controls were prepared by placing tubes in a boiling water bath prior to incubation and the contents carried through the entire procedure. The sample contents of each tube were transferred to a dialysis sac and dialyzed at 4 C against distilled water for 24 hr. The cellulose membrane had been boiled in distilled water twice prior to use. In other experiments the membrane contents were adjusted to 20 mM EDTA prior to dialysis. After measuring the volume within the sac, sample aliquots were removed, concentrated, 15 ml scintillation solution was added, and radioactivity was measured using a Packard Liquid Scintillation Spectrometer (Model 3380).

The data obtained from enzymatic digestion and dialysis of UDP-[^{14}C]-galactose and [^{3}H]-G_{M1} are presented in Table I. It is apparent that EDTA addition alone enhances the loss of nucleotide sugar upon dialysis and that enzymatic digestion followed by dialysis removes virtually all of the nucleotide sugar. Identical results were obtained when UDP-[^{14}C] glucose was substituted for UDP-[^{14}C]-galactose.

Analysis of the [^{3}H]-G_{M1} data (Table I) reveals that approximately 95% of the ganglioside can be recovered after dialysis, provided sufficient carrier ganglioside was added to exceed the critical micelle concentration. Attempts to increase the recovery of [^{3}H]-G_{M1} further by increasing the concentration of carrier ganglioside or pretreating the membrane with boiling EDTA solutions were unsuccessful.

B. High Voltage Paper Electrophoresis

The incubation mixture employed by Kaufman et al. (4) was adopted to evaluate this technique. This mixture contained 200 μg Tween 80, 400 μg Triton CF-54, 20 μmol cacodylate-HCl buffer (pH 6.3), 2.0 μmol $MgCl_2$, and 860 μg bovine serum albumin in a final volume of 200 μl. After the addition of [^{3}H]-G_{M1} (11.0 nmol) or UDP-[^{14}C]-galactose (0.054 nmol), the entire reaction mixture was streaked on Whatman 3 MM chromatographic paper (46 x 57 cm). The paper was saturated with buffer and electrophoresed for 30 min at 55 volts/cm (230 ma) employing 1% sodium tetraborate (pH 9.0) as buffer and a model D-2 Gilson Electrophorator. The chromatogram was air-dried and the radioactivity located by employing a Berthold radio scanner.

In additional experiments, various concentrations of non-radioactive mixed bovine brain gangliosides or G_{M1} and G_{M2} were subjected to electrophoretic separation, and the paper was divided into 2 cm strips. The individual strips were soaked in $CHCl_3$-MeOH (2:1) to elute the gangliosides. The eluate was concentrated to dryness, dissolved in a small volume of solvent, and the gangliosides were analyzed by TLC employing $CHCl_3$-MeOH-2.5 N NH_4OH (60:35:8) as the developing solvent. The gangliosides were detected by use of resorcinol-HCl reagent (16). This TLC and detection system was employed throughout these studies, unless specifically stated otherwise.

A radioscan of [^{3}H]-G_{M1} and UDP-[^{14}C]-galactose, after paper electrophoresis employing sodium tetraborate as buffer, is presented in Figure 1. This monosialoganglioside remained close to the origin, 0-6 cm, while the nucleotide sugar migrated near the solvent front, providing good separation of the two materials. When mixtures of 25 to 300 nmol of bovine brain gangliosides were subjected to such electrophoretic separation, the gangliosides were found streaked to approximately 14 cm from the origin. This observation was documented by TLC analysis of the $CHCl_3$-MeOH eluates from the paper as shown in Figure 2. Similar results

TABLE I

Dialysis of Nucleotide Sugar and Ganglioside: Effect of EDTA and Enzyme Treatment

	% Loss of UDP-^{14}C-galactose[d]	% Loss of ^{3}H-G_{M1}[e]
Direct dialysis[a]	57.0	5.7
Plus EDTA[b]	92.6	4.3
Plus EDTA[b] and enzyme treatment[c]	99.8	5.1

[a]Samples dialyzed directly against 500 volumes distilled H_2O, final ganglioside concentration after the addition of carrier was 4 x 10^{-4} M.

[b]Contents of dialysis sac made 20 mM in EDTA prior to dialysis.

[c]Snake venom phosphodiesterase and bacterial alkaline phosphatase treatment of samples prior to dialysis.

[d](0.131 nmol).

[e](11.0 nmol).

were obtained when G_{M1} and G_{M2} were subjected to this electrophoretic procedure.

C. Ascending Paper Chromatography

The procedure employed for these studies was identical to that in B above, except that separation was achieved with ascending paper chromatography rather than electrophoresis and UDP-[^{14}C]-glucose was substituted for UDP-[^{14}C]-galactose. After 1.5 hr, the paper was air-dried and a radioscan obtained. When bovine brain gangliosides were utilized, the paper chromatogram was cut into 3 equal areas, and similar control sections were obtained from

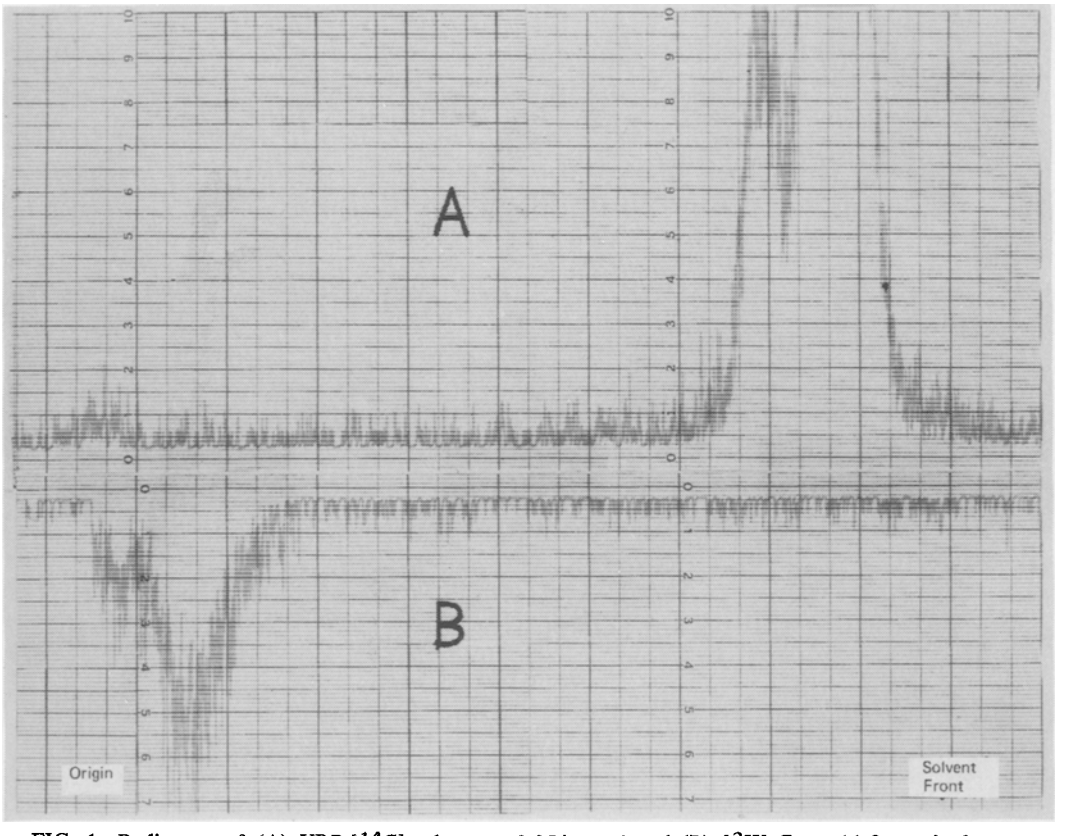

FIG. 1. Radioscan of (A) UDP-[^{14}C]-galactose, 0.054 nmol and (B) [^{3}H]-G_{M1}, 11.0 nmol after paper electrophoresis. Entire incubation mixture was streaked on Whatman 3 MM chromatographic paper (46 x 57 cm). After paper electrophoresis, employing 1% sodium tetraborate (pH 9.0) and 55 volts/cm (230 milliamperes) for 30 min, chromatogram was air dried and analyzed.

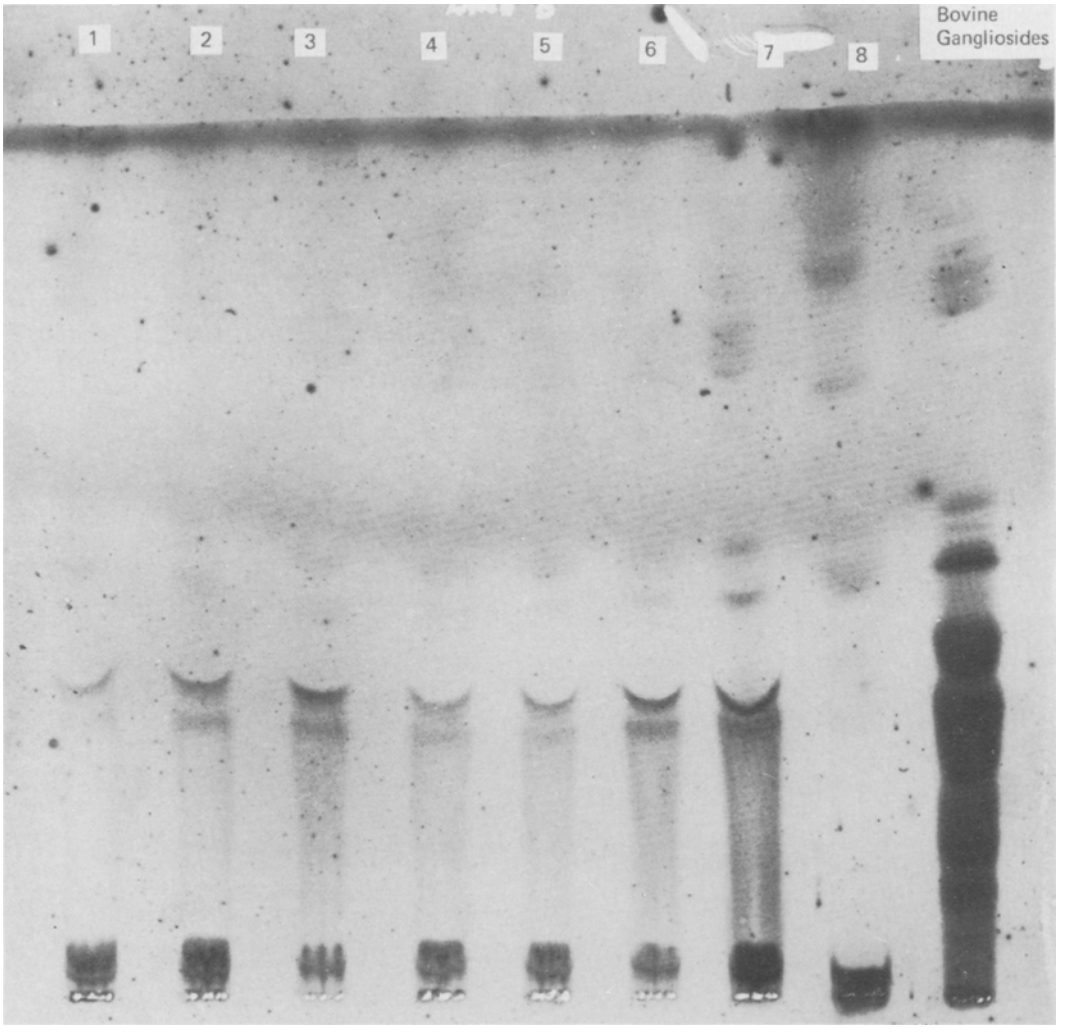

FIG. 2. TLC analysis of sections of a chromatogram after high voltage electrophoresis of mixed brain gangliosides. Experimental conditions were the same as those outlined in Figure 1, except that 100 nmol of bovine brain gangliosides were substituted for [^{3}H]-G_{M1}. After electrophoresis, paper was sectioned into 2 cm strips, except 8 which included remainder of chromatogram. TLC lanes are numbered 1 to 9 from left to right. Lane 1 includes origin region, while lanes 2 through 7 represent 2 cm strips of chromatogram from 2 to 4, 4 to 6, 6 to 8, 8 to 10, 10 to 12, and 12 to 14 cm from origin. Lane 9 represents a mixture of bovine brain gangliosides not subjected to high voltage electrophoresis. Gangliosides were eluted from the paper with $CHCl_3$-MeOH (2:1).

lanes devoid of sample. Various sections of the chromatogram were extracted with $CHCl_3$-MeOH (2:1), and aliquots were taken for both quantitative colorimetric analysis of sialic acid using the resorcinol-HCl method of Miettinen and Takki-Luukkainen (16) and TLC.

A radioscan of [^{3}H]-G_{M1} and UDP-[^{14}C]-glucose, after ascending paper chromatography, is presented in Figure 3. Less than 5% of the total G_{M1} applied stayed at the origin, while the remainder cochromatographed with UDP-[^{14}C]-glucose, close to the solvent front. Similar results were obtained with a mixture of bovine brain gangliosides, as shown in Figure 4. Nearly all of the gangliosides applied to the paper migrated at or near the solvent front. Sialic acid determination from the various regions of the chromatogram also confirmed these observations.

D. Direct TLC

UDP-galactose, UDP-glucose, N-acetylgalactosamine, N-acetylneuraminic acid and a mixture of bovine brain gangliosides were spotted on a 0.25 mm precoated Silica Gel G plate and developed with $CHCl_3$-MeOH-2.5 N NH_4OH,

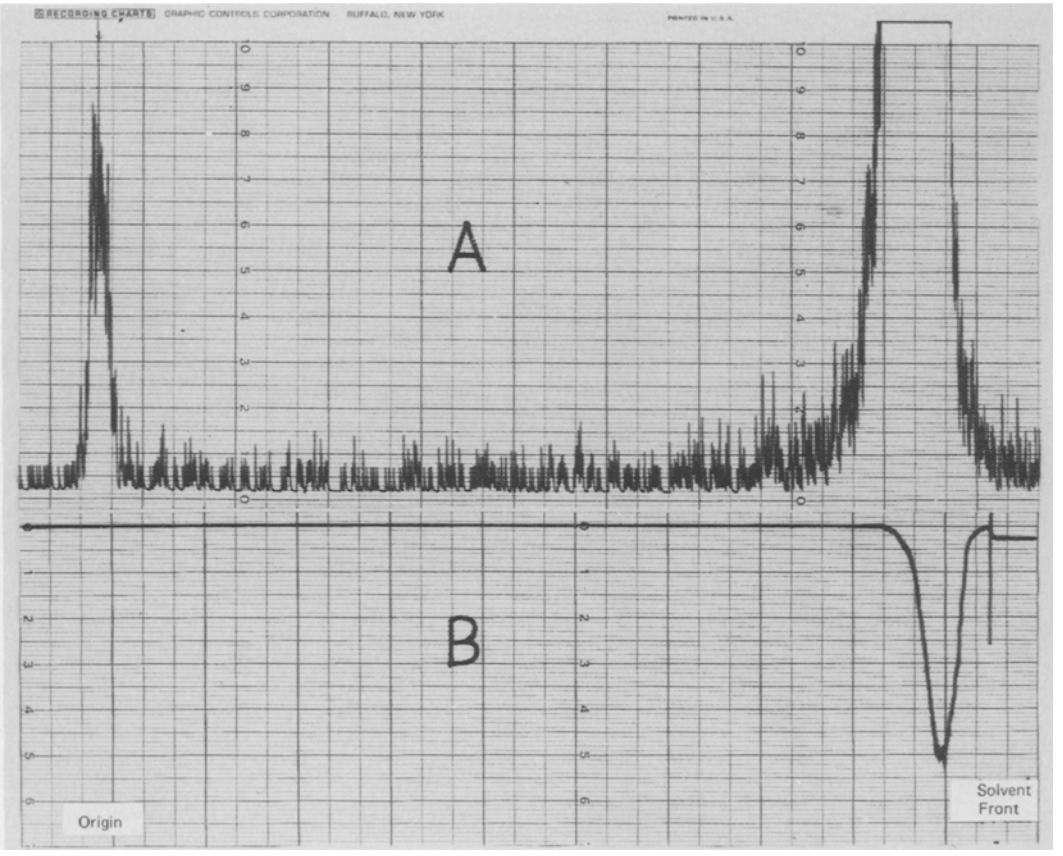

FIG. 3. Radioscan of (A) [^{3}H]-G_{M1}, 11.0 nmol and (B) UDP-[^{14}C]-glucose, 0.058 nmol after ascending paper chromatography. Entire reaction mixture was streaked on Whatman 3 MM chromatographic paper and developed with 1% (w/v) sodium tetraborate (pH 9.0) as described in the text. After development (1.5 hr), paper was air dried and analyzed.

60:35:8 (17). Components were visualized by spraying the plate lightly with 50% H_2SO_4 and heating for 30 min at 120 C.

Direct TLC of the nucleotide sugars, various ganglioside precursors and a mixture of bovine brain gangliosides is represented in Figure 5. Since these monosaccharides and nucleotide sugars have R_f values similar to those of the more polar gangliosides, separation of gangliosides from these constituents cannot be accomplished by such routine TLC separation.

E. Sephadex Column Chromatography

Pretreatment of the Sephadex G-25 to remove fines, solvent volumes and eluting parameters employed were according to the procedure of Wells and Dittmer (6). In vitro conditions were simulated by using 200 μl of incubation mixture I and 3 μl [^{3}H]-G_{M1} (6.6 nmol) or 2 μl UDP-[^{14}C]-galactose (0.054 nmol). The sample was applied to the column (1.0 x 20 cm), after the addition of 10 ml of $CHCl_3$-MeOH-H_2O (60:30:4.5), and fractions were collected and assayed for radioactivity. Similar experiments also were performed with a mixture of bovine brain gangliosides and UDP-[^{14}C]-galactose or UDP-[^{14}C]-glucose. In this case aliquots from the column fractions also were taken for sialic acid determination (16).

The [^{3}H]-G_{M1} applied to a Sephadex column was eluted in the $CHCl_3$-MeOH-H_2O (60:30:4.5) fraction or in the $CHCl_3$-MeOH (2:1) fractions as shown in Table II. In contrast, all of the UDP-[^{14}C]-galactose was recovered in the MeOH-H_2O (1:1) fractions, indicating that gangliosides can be separated easily from nucleotide sugars by this procedure. Experiments using a mixture of bovine brain gangliosides and labeled UDP-galactose or UDP-glucose confirmed this observation. However, only 75% of the [^{3}H]-G_{M1} was recovered from the Sephadex column. Similar recoveries of gangliosides also were obtained when bovine brain gangliosides were subjected to this procedure.

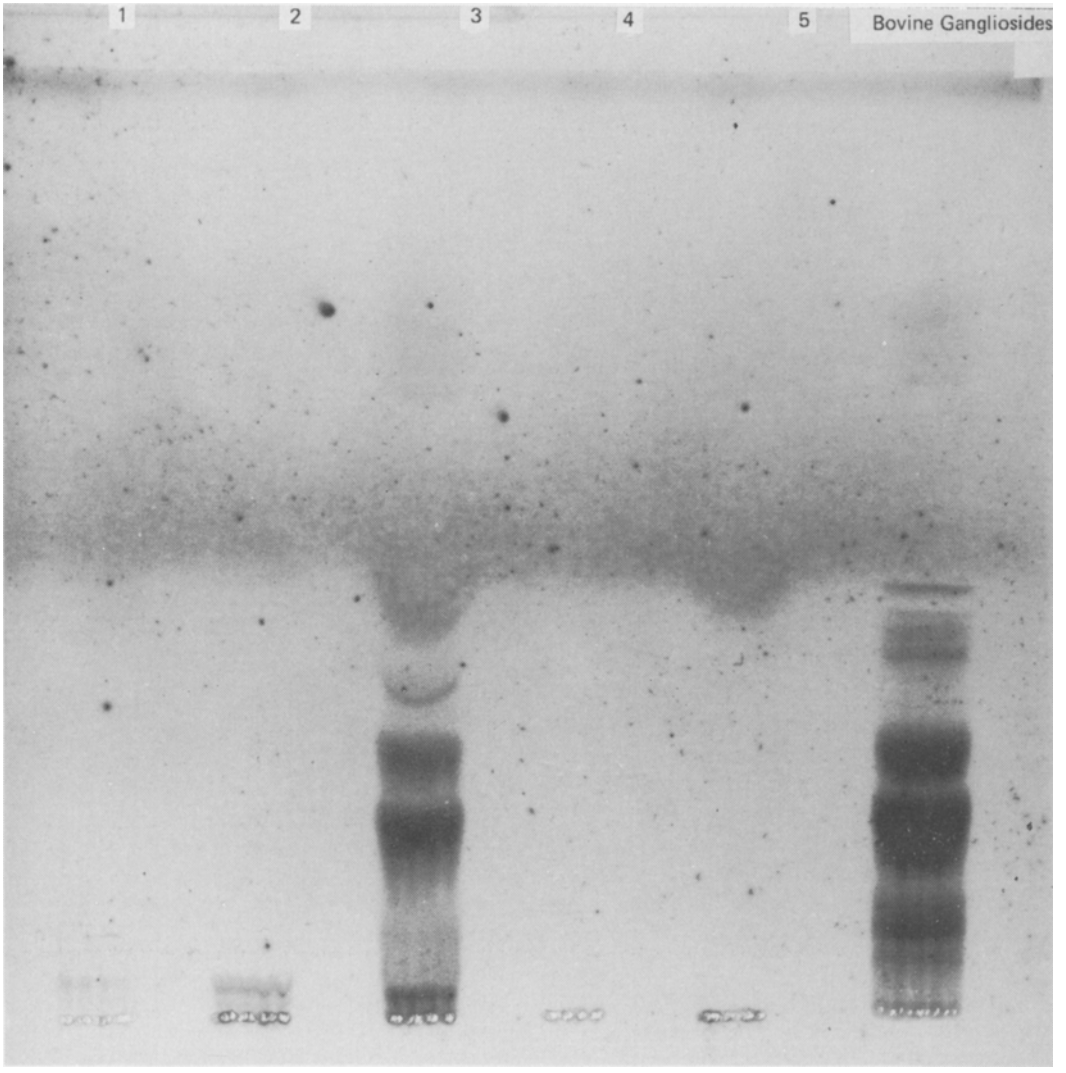

FIG. 4. TLC analysis of ganglioside eluted from sections of chromatogram after ascending paper chromatography. Experimental conditions were the same as those outlined in Figure 3, except 100 nmol of a mixture of bovine brain gangliosides was employed. After development, paper was air dried and sectioned into thirds. Lanes are numbered from left to right; lane 1 includes origin region, lane 2 the middle 1/3, and lane 3 the upper 1/3. Lane 4 is a control of equal area from the middle of the chromatogram which did not contain any sample. Lane 5 is a control from the solvent front region; lane 6 represents a mixture of bovine brain gangliosides not subjected to ascending paper chromatography. Gangliosides were eluted from the paper with $CHCl_3$-MeOH (2:1); solutions were concentrated and analyzed by TLC.

F. Silicic Acid Column Chromatography (7)

The column (1.5 cm ID) was packed with 10 gm of prewashed SilicAR suspended in $CHCl_3$-MeOH- H_2O (65:25:4). Four milliliters of a $CHCl_3$-MeOH (2:1) solution containing 88 mg total mixed calf brain lipid and 5 μl UDP-[^{14}C]-glucose (0.058 nmol) were transferred to the column. The column was eluted with 100 ml $CHCl_3$-MeOH -H_2O (65:25:4) followed by 300 ml $CHCl_3$-MeOH-H_2O (60:35:8) at a flow rate of 3.5 ml/min. Individual 20 ml fractions were collected, and aliquots were taken for analysis of gangliosides by TLC and for analysis of nucleotide sugars by radioactivity.

The data obtained from fractionation of nucleotide sugar on silicic acid columns indicated that the majority of the UDP-[^{14}C]-glucose applied was eluted with the first 80 ml of $CHCl_3$-MeOH-H_2O (60:35:8). TLC analysis of gangliosides from column fractions revealed that the majority of bovine brain gangliosides

TABLE II

Separation of ^{3}H-G_{M1} and ^{14}C-UDP-galactose by Sephadex G-25 Column Chromatography

Eluted fractions	Solvent	cpm	
		Column I ^{3}H-G_{M1}[d]	Column II ^{14}C-UDP-galactose[e]
1	10 ml solvent I[a]	2043	ND[f]
2	2 ml solvent II[b]	998	ND
3	2 ml solvent II	249	ND
4	2 ml solvent II	171	ND
5	2 ml solvent II	125	ND
6	2 ml solvent II	37	21
7	10 ml solvent III[c]	18	29
8	5 ml solvent III	18	57
9	5 ml solvent III	ND[f]	16931
10	5 ml solvent III	ND	17387
11	5 ml solvent III	ND	15
cpm recovered		3659	34440
cpm-applied		4831	34490
	% Recovery	75.7	99.9

[a]$CHCl_3$-MeOH-H_2O (60:30:4.5).
[b]$CHCl_3$-MeOH (2:1).
[c]MeOH-H_2O (1:1).
[d]6.6 nmol.
[e]0.054 nmol.
[f]Not detected.

also eluted with the first 80 ml of this solvent.

G. TCA Coprecipitation of Ganglioside and Bovine Serum Albumin

Treatment I: Carrier bovine serum albumin (BSA) (1-5 mg) was added to 200 μl of incubature mixture I, which contained 5 μl [^{3}H]-G_{M1} (11.0 nmol) or 2 μl UDP-[^{14}C]-glucose (0.058 nmol). To this mixture 1 ml of 10% trichloroacetic acid was added, the tubes were mixed and centrifuged. The supernatant was decanted, 6 ml of $CHCl_3$-MeOH (2:1) was added and the mixture agitated. The contents of the tubes were soluble in $CHCl_3$-MeOH (2:1) provided the final BSA concentration did not exceed 0.7 mg/ml. Aliquots of solutions, as well

TABLE III

Effect of Various Cations, Lipid and H^+ Concentration on Distribution of ^{3}H-G_{M1} After Extraction of a TCA Precipitate[a]

Aqueous extraction media		^{3}H-G_{M1} Distribution (%)	
		(pH 4.0[c])	(pH 7.0[d])
Control (-salt, -lipid)	Upper phase	57	77
	Lower phase	43	23
1 M KCl	Upper phase	32	29
	Lower phase	68	71
1 M NaCl	Upper phase	28	31
	Lower phase	72	69
Lipid[b]	Upper phase	36	40
	Lower phase	64	60
400 mM $CaCl_2$	Upper phase	19	50
	Lower phase	81	50
100 mM $CaCl_2$	Upper phase	16	48
	Lower phase	84	52

[a]Carrier BSA, 4 mg.
[b]TCA precipitate extracted with $CHCl_3$MeOH (2:1) containing 120 mg of crude bovine brain lipid and ganglioside partitioned with buffer.
[c]Potassium biphthalate buffer, 0.05 M.
[d]Sodium phosphate buffer, 0.2 M.

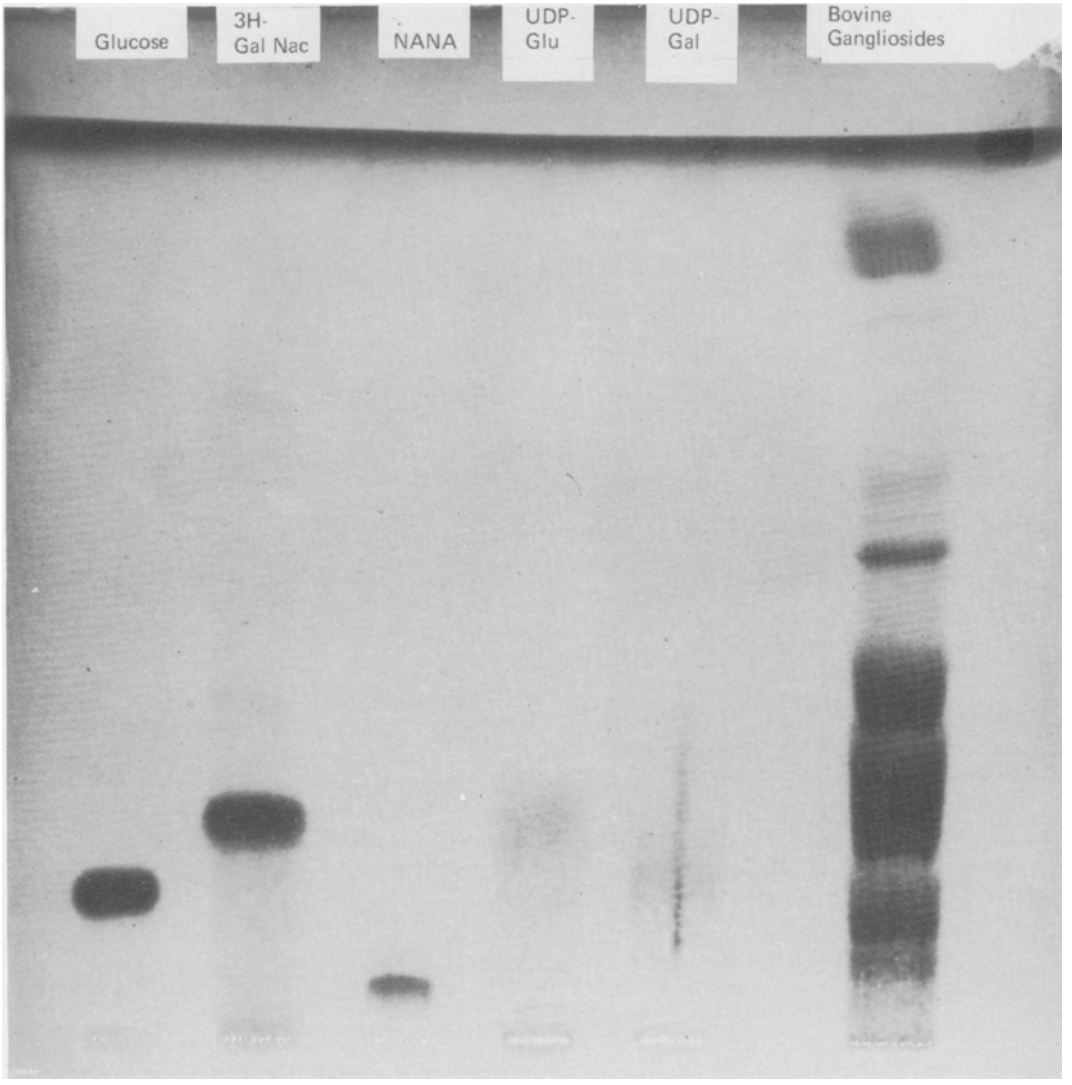

FIG. 5. Direct TLC of ganglioside precursors, lane 1 glucose, lane 2 N-acetylgalactosamine, lane 3 N-acetylneuraminic acid, lane 4 UDP-glucose, lane 5 UDP-galactose, and lane 6 a mixture of bovine brain gangliosides. Lanes are numbered from left to right. TLC support was Silica Gel G (250 microns) and developing solvent was $CHCl_3$-MeOH-2.5 N NH_4OH (60:35:8). Compounds were visualized after spraying plate with 50% H_2SO_4 and heating for 30 min at 120 C.

as appropriate controls (no trichloroacetic acid treatment), were taken for analysis of ganglioside and nucleotide sugar by conventional liquid scintillation methods.

The results of trichloroacetic acid coprecipitation of gangliosides with various concentrations of added carrier BSA are presented in Figure 6. Under these conditions the maximum recovery of [^{3}H]-G_{M1}, 82%, was obtained when 4 mg of BSA was present. Duplicate assays often showed 5-10% differences in the recovery of [^{3}H]-G_{M1}. Additional experiments indicated that increasing the concentration of either trichloroacetic acid or BSA and the volume of $CHCl_3$-MeOH (2:1) employed did not increase the recovery of ganglioside or decrease the error associated with duplicate analyses. Under these conditions, ca. 10% of the radioactivity from UDP-[^{14}C]-glucose or UDP-[^{14}C]-galactose initially present was recovered in the $CHCl_3$-MeOH (2:1) solution.

Treatment II: The same procedure, as outlined in treatment I, was employed, except the $CHCl_3$-MeOH solubilized trichloroacetic acid precipiate with 4 mg carrier BSA was extracted once with 1.5 ml of various salt solutions which

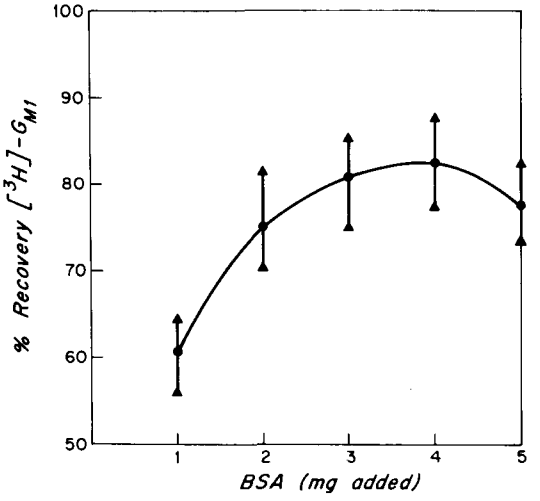

FIG. 6. Per cent [^{3}H]-G_{M1} recovered as function of BSA concentration when ganglioside and protein were coprecipitated with 10% trichloroacetic acid. Varying amounts of carrier BSA were added to 200 μl of incubation mixture I, which contained 5 μl [^{3}H]-G_{M1} (11.0 nmol). After 10% trichloroacetic acid (1.0 ml) was added, contents of tube were mixed well, centrifuged, and supernatant discarded. Precipitate was dissolved in 6.0 ml $CHCl_3$-MeOH (2:1), and aliquots were taken for quantitative determination of radioactivity. • averages of three experiments, ▲——▲ range of values.

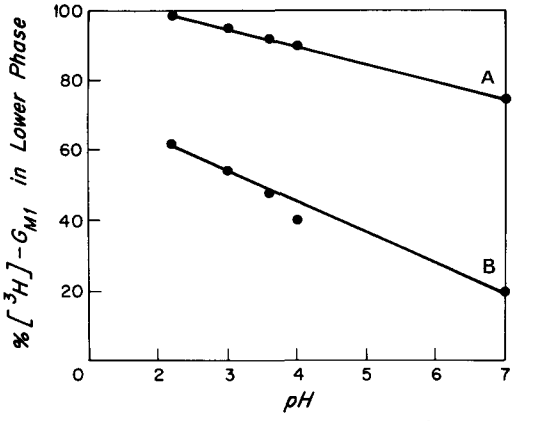

FIG. 7. Effect of pH on distribution of [^{3}H]-G_{M1} between the two phases. To 200 μl incubation mixture I, 5 μl [^{3}H]-G_{M1} (11.0 nmol) and 6 ml $CHCl_3$-MeOH (2:1) were added. Contents of individual test tubes then were extracted with 1.5 ml 0.2 M buffer containing 100 mM CaCl. Buffers employed were 0.2 M glycine-HCl at pH 2.2, pH 3.0 or pH 3.6, 0.05 M potassium biphthalate at pH 4.0 and 0.2 M sodium phosphate at pH 7.0. Controls were extracted with similar buffered solutions which did not contain $CaCl_2$. After centrifugation, individual upper and lower phases were analyzed for ganglioside by determining radioactivity. A, 100 mM $CaCl_2$; B, control.

included either 1 M NaCl, or 1 M KCl, or 100 mM $CaCl_2$, or 400 mM $CaCl_2$. To determine whether pH changes had any effect on the ganglioside partitioning, the salt solutions were prepared in either 0.05 M potassium biphthalate buffer at pH 4.0 or 0.2 M sodium phosphate buffer at pH 7.0. The effect of added non-ganglioside lipid was studied by dissolving the trichloroacetic acid insoluble material in 6 ml $CHCl_3$-MeOH (2:1) containing 120 mg bovine brain lipids prior to extraction with buffer in the absence of added salt. After extraction and centrifugation, aliquots of the individual upper and lower phases were taken for counting to determine quantitatively the distribution of [^{3}H]-G_{M1} or UDP-[^{14}C]-glucose.

The results of experiments on the effect of various cations, pH, and added non-ganglioside lipid on the partitioning of [^{3}H]-G_{M1} into the upper and lower phase after trichloroacetic acid treatment are presented in Table III. The presence of either K^+, Na^+, Ca^{++}, or lipid enhances the partitioning of [^{3}H]-G_{M1} into the chloroform-rich lower phase, while an increase in the H^+ concentration causes a similar effect only in the control or in the presence of Ca^{++}. At pH 4.0 in the presence of 100 mM $CaCl_2$, 84% of the ganglioside was found in the lower phase or at the interphase. In all of these partitioning experiments, less

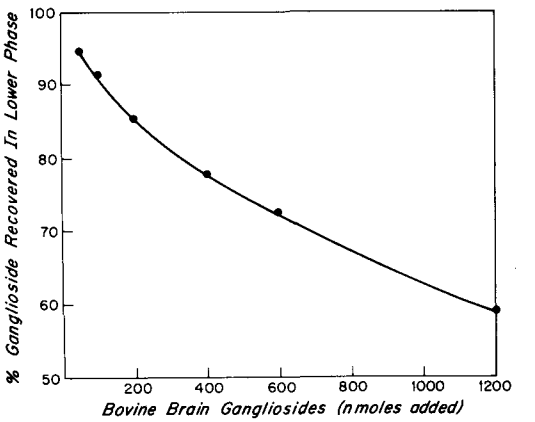

FIG. 8. Per cent recovery of ganglioside sialic acid in the lower phase after four extractions with buffered $CaCl_2$. To 200 μl incubation mixture I, 5 to 120 μl of a $CHCl_3$-MeOH (2:1) solution containing 10 nmol of mixed bovine brain gangliosides/μl and 6 ml $CHCl_3$-MeOH (2:1) were added. Contents of individual test tubes were extracted 4 times with 1.5 ml 0.2 M glycine-HCl buffer at pH 2.2, containing 100 mM $CaCl_2$. Methanol (1 ml) was added to lower phases, and aliquots from individual lower and combined upper phases were taken for analysis of sialic acid (18). Points represent the average of two experiments.

than 1% of the radioactivity from nucleotide sugars was recovered in the lower phases.

H. Direct Folch Partitioning: Effect of $CaCl_2$ and H^+ Concentration

Treatment I. To 200 μl incubation mixture I

TABLE IV

Effect of Repeated Extraction of Lower Phase with 100 mM $CaCl_2$ at pH 2.2[a] on Distribution of ^3H-G_{M1}, ^3H-N-acetyl-galactosamine and ^{14}C-UDP-glucose[b]

Number of extractions		(% Distribution of constituent)			
		1	2	3	4
^3H-G_{M1}					
	Upper phase (s)	1.0	2.3	3.0	3.5
	Lower phase	99.0	97.7	97.0	96.5
^3H-N-acetyl-galactosamine					
	Upper phase (s)	82.3	91.0	95.8	98.3
	Lower phase	17.7	9.0	4.2	1.7
^{14}C-UDP-glucose					
	Upper phase (s)	81.0	90.9	96.0	98.0
	Lower phase	19.0	9.1	4.0	2.0

[a]Glycine-HCl buffer, 0.2 M.

[b]Experimental conditions were same as those outlined in Figure 7, except individual lower phases were extracted 1-4 times with 1.5 ml 100 mM $CaCl_2$ (pH 2.2).

containing 5 μl [^3H]-G_{M1} (11.0 nmol), 6 ml $CHCl_3$-MeOH (2:1) was added. The contents of the individual test tubes then were extracted with 1.5 ml of 10-400 mM $CaCl_2$ solution and centrifuged. In similar experiments, the $CHCl_3$-MeOH solutions were extracted with 1.5 ml of 100 mM $CaCl_2$ solutions buffered with 0.2 M glycine-HCl at pH 2.2, pH 3.0 or pH 3.6, 0.05 M potassium biphthalate at pH 4.0 or 0.2 M sodium phosphate at pH 7.0. The individual upper and lower phases were analyzed for ganglioside by determining radioactivity. The insoluble interphase material which appeared during the partitioning always was combined with the lower phase and rendered soluble by the addition of a small amount of MeOH. This was done because initial experiments indicated that gangliosides were distributed equally between the interphase and the lower phase after extraction with Ca^{++} solutions.

The optimal Ca^{++} concentration for partitioning gangliosides into the chloroform-rich lower phase was found to be 100 mM. The H^+ concentration of the extraction medium had a dramatic effect upon the distribution of [^3H]-G_{M1} in the presence of 100 mM $CaCl_2$ as shown in Figure 7. Such a pH effect agrees with earlier observations (8). It is evident from these data that 99% of the [^3H]-G_{M1} was excluded from the upper phase when glycine-HCl buffer (pH 2.2) containing 100 mM $CaCl_2$ was employed.

Treatment II: Either 5 μl [^3H]-G_{M1} (11.0 nmol), 10 μl [^3H]-N-acetylgalactosamine (6.25 nmol), or 10 μl UDP-[^{14}C]-galactose (0.29 nmol) was added to 200 μl of incubation mixture I. After the addition of 6 ml $CHCl_3$-MeOH (2:1) to each tube, the contents were extracted 1 to 4 times with 1.5 ml cold 0.2 M glycine-HCl buffer, pH 2.2, containing 100 mM $CaCl_2$. Aliquots of both the upper and lower phase were removed and radioactivity determined by liquid scintillation counting.

The effect of varying the concentration of a mixture of bovine brain gangliosides on the distribution of lipid-bound sialic acid was investigated by employing 50 to 1200 nmol (5-120 μl) ganglioside. These solutions were subjected to 4 extractions with the above buffered salt solution. In these experiments the individual lower phase sialic acid content as well as that in the combined upper phases was determined by the thiobarbituric acid method of Aminoff (18).

Repeated extractions of the ganglioside-containing lower phase indicated that over 95% of this material remained in the lower phase, while 98% of either the [^3H]-N-acetyl-galactosamine or the UDP-[^{14}C]-glucose was removed from this simulated in vitro mixture under these conditions (Table IV). The results of experiments utilizing 50 to 1200 nmol bovine brain gangliosides employing 4 extractions with 100 mM $CaCl_2$, pH 2.2, indicated that the recovery of sialic acid in the lower phase was greater than 85% for concentrations less than 200 nmol, as presented in Figure 8.

Treatment III: To the final washed lower phases from treatment II which contain 30 to 200 nmol of a mixture of bovine brain gangliosides, solvent was added to reconstitute a $CHCl_3$-MeOH ratio of 2:1. The contents of the individual test tubes (total volume 9.6 ml) were then extracted once with 2.4 ml 0.1 M EDTA (pH 7.0) and 4 times with 2.0 ml theoretical upper phase containing 0.1 M EDTA (pH 7.0) instead of H_2O. The individual lower phases and pooled upper phases were concentrated to

dryness, the gangliosides solubilized by vigorous extraction of the residue with $CHCl_3$-MeOH (2:1), and aliquots were taken for determination of sialic acid (16) and TLC analysis.

The data from experiments where the gangliosides were partitioned from the lower phase into the upper phase are represented graphically in Figure 9. The results indicate that, for ganglioside concentrations less than 80 nmol, greater than 85% of the ganglioside can be recovered in the upper phase. Comparison of this figure with Figure 8 reveals that there is a 5% loss of ganglioside associated with attempts to repartition gangliosides into the upper phase when employing this EDTA procedure. TLC analysis of bovine brain gangliosides subjected to this procedure revealed that there were no selective losses of individual ganglioside species.

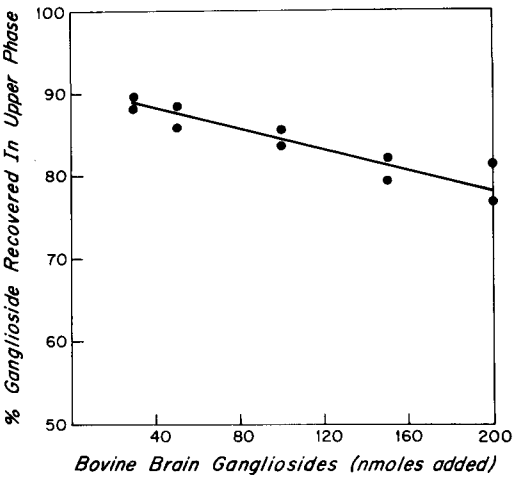

FIG. 9. Effect of ganglioside concentration on their partitioning from the lower phase into the upper phase. To the starting ganglioside-containing lower phase solutions (obtained as described in Figure 8), solvent was added to reconstitute a $CHCl_3$-MeOH ratio of 2:1, and individual test tube contents (total volume 9.6 ml) were extracted once with 2.4 ml 0.1 M EDTA, pH 7.0 and 4 times with 2.0 ml theoretical upper phase containing 0.1 M EDTA, pH 7.0, instead of H_2O. Upper phases were pooled, concentrated to dryness, gangliosides solubilized by vigorous extraction of the residue with $CHCl_3$-MeOH (2:1), and aliquots taken for determination of sialic acid content (16). Each point represents the average of values obtained from a single experiment.

DISCUSSION

Several of the previously employed techniques for the removal of water-soluble nucleotide sugar contaminants from gangliosides in reconstituted in vitro incubation mixtures were examined. Streaking of gangliosides occurred with more than 25 nmol when the electrophoretic method was employed, and the inability to separate these glycosphingolipids from nucleotide sugars was observed with the paper chromatographic procedure. Although the results of TLC analyses indicate that there are selective losses of individual ganglioside species when employing either of these methods, it is likely that such losses are due to the incomplete elution of these lipids from the paper chromatogram.

The results of Sephadex and silicic acid column chromatography indicate that these methods have limitations for in vitro ganglioside studies. The silicic acid column chromatographic procedure is able to separate gangliosides from other lipids, but it is unsatisfactory for the removal of nucleotide sugars from ganglioside preparations. The Sephadex G-25 procedure is capable of eliminating virtually all of the nucleotide sugars from ganglioside mixtures, which agrees with the report of Wells and Dittmer (6). However, ca. 25% of the applied ganglioside is retained by this dextran gel. In addition, it is quite tedious and time consuming for a routine in vitro assay system.

Similar losses also are obtained when gangliosides and protein are coprecipitated with trichloroacetic acid. Tallman and Brady (19) recently reported that their TCA supernatant blank values for boiled enzyme were ca. one-fourth of the values of incubations with active sialidase when [NeuAc-^{3}H]-G_{M2} was used as substrate. In addition to a 20-25% loss of ganglioside, ca. 10% of the nucleotide sugar was found in the trichloroacetic acid precipitate.

Of the reported methods investigated, enzyme digestion followed by dialysis proved to be the best technique, since virtually all the nucleotide sugar can be excluded with minimal loss of gangliosides. However, the necessity of adding carrier ganglioside makes this method undesirable, since neither detailed analyses of individual ganglioside species produced in vitro nor determination of specific activities of the individual gangliosides is possible.

A procedure for removal of water-soluble constituents from simulated ganglioside in vitro incubation mixtures is based upon previous observations by a number of workers (8-10). This method takes advantage of the lipophilic character of these glycosphingolipids when ionization of the sialic acid residues is depressed by low H^+ concentrations resulting in the formation of water insoluble ganglioside-Ca^{++} complexes. The formation of lipophilic ganglioside calcium salts was reported by both Quarles and Folch-Pi (9) and Gatt (8). However, the chemical structures of these complexes were not documented. Purification of gangliosides from in vitro preparations by this technique offers

several advantages: (a) This method is easily adaptable for routine analyses and is not as tedious as other procedures. (b) Removal of virtually all contaminating water-soluble constituents is possible with minimal loss of ganglioside, and (c) the unaltered individual ganglioside species can be recovered for detailed analyses.

In actual in vitro situations this method was found to be as effective as reported above. Blanks never yielded radioactive precursors exceeding 0.4% of the amount added. However, the addition of a small amount of bovine-white-matter non-ganglioside lipid was found to be mandatory where the lipid content of the individual incubation mixture was less than 800 μg prior to Folch partitioning by this method. Aside from in vitro studies it is possible that this procedure also could be utilized when isolating small quantities of gangliosides and when purification of these glycosphingolipid preparations by dialysis is not possible, e.g. non-neural and subcellular organelle studies.

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REFERENCES

1. Kanfer, J.N., and R.O. Brady, in "Inborn Disorders of Sphingolipid Metabolism," Edited by S.M. Aronson and B.W. Volk, Pergamon Press, New York, 1967, p. 187.
2. Gammack, D.B., J. Biochem. (Tokyo) 88:373 (1963).
3. Kanfer, J.N., and C.H. Spielvogel, J. Neurochem, In press (1973).
4. Kaufman, B., S. Basu and S. Roseman, in "Inborn Disorders of Sphingolipid Metabolism," Edited by S.M. Aronson and B.W. Volk, Pergamon Press, New York, 1967, p. 193.
5. Kaufman, B., S. Basu and S. Roseman, in "Methods in Enzymology," Edited by S.P. Colowick and N.O. Kaplan, Vol. VIII, Academic Press, New York, 1966, p. 365.
6. Wells, M.A., and J.C. Dittmer, Biochem. 2:1259 (1963).
7. Holm, M., and L. Svennerholm, J. Neurochem. 19:609 (1972).
8. Gatt, S., J. Neurochem. 12:311 (1965).
9. Quarles, R., and J. Folch-Pi, J. Neurochem. 12:543 (1965).
10. Kruger, S., and M. Mendler, J. Neurochem. 17:1313 (1970).
11. Svennerholm, L., J. Lipid Res., 5:145 (1964).
12. Folch-Pi, J., M. Lees and G.H. Sloane-Stanley, J. Biol. Chem. 226:497 (1957).
13. Winterbourn, C.C., J. Neurochem. 18:1153 (1971).
14. Radin, N.S., L. Hof, R.M. Bradley and R.O. Brady, Brain Res. 14:497 (1969).
15. Brunetti, P., G.W. Jourdian and S. Roseman, J. Biol. Chem. 237:2447 (1962).
16. Miettinen, T., and L-T Takki-Luukkainen, Acta Chem. Scand. 13:356 (1959).
17. Renick, R.J., M.H. Meisler and R.H. McCluer, Biochem. Biophys. Acta 116:279 (1966).
18. Aminoff, D., Biochem. J. 81:384 (1961).
19. Tallman, J.F., and R.O. Brady, J. Biol. Chem. 247:7570 (1972).

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Inhibition of Staphylococcus Aureus Lipase Activity by Alcohols

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ABSTRACT

A series of saturated *n*-primary alcohols has been examined for its inhibitory effect on staphylococcal lipase. The efficiency of the inhibition of hydrolysis of the triglycerides increased with the chain length of the alcohol and reached a maximum of seven carbon atoms. Further increases in the chain length caused a decrease in the inhibitory ability of the alochol. However the efficiency of the inhibitory effect on Tween 80 hydrolysis increased with the chain length up to 12 carbon atoms. The degree of inhibition caused by the secondary alcohols decreased with the removal of the hydroxyl group away from the end of the chain. The similar keto compounds were more effective than were the alochol compounds. The effect of the alcohols on the enzyme activity could be overcome by addition of more substrate. Evidence is presented that shows that the inhibiting activity of the alcohol is due to its adsorption onto the substrate, thus blocking the enzyme from the substrate.

INTRODUCTION

Lipase (E.C. 3.1.1.3) has been found to hydrolyze esters, which are usually water insoluble, at the interphase between the water and lipid (1). The most typical substrates for the action of this enzyme are naturally occurring triglycerides containing various fatty acids. Entressangles and Densnuelle (2) have shown that pancreatic lipase can also act on what appeared to be a single phase of triacetin, tripropionin and 1,3 dibutyrin in 0.1M NaCl. The rate of the enzymatic hydrolysis appears to depend on the concentration of triglyceride micelles formed under these conditions. Brockerhoff (3) continued this work and has shown that the activity of hog pancreatic lipase against emulsified esters decreases with the increased solubility of the substrate in water. Soluble esters can competitively inhibit the lipolysis of emulsified tripropionin. A similar inhibition can be found with water soluble alcohols and ethers. Recently Mathson et al. (4) have shown that *n*-alcohols inhibit the hydrolysis of methyl oleate by pancreatic lipase.

In a previous publication (5) we have reported that the lipase produced by *Staphylococcus aureus* can split not only triglycerides but also tweens, which are soluble in water. The purpose of this work was to investigate the effect of alochols, aldehydes and ketones on staphyloccal lipase activity when using soluble and insoluble substrates.

MATERIALS AND METHODS

Organism

Staphylococcus aureus 111 produced a lipase that was able to hydrolyze seven triglycerides and five tweens (polyoxyethylene sorbitan-fatty acid) (5). The bacteria were kept on trypticase soy agar (BBL) and were transferred twice a week. The organism was incubated at 37 C for 18 hr in trypticase soy broth (BBL), and 1 ml of this culture was inoculated into a 2 liter erlenmeyer flask containing 1 liter of the same medium. The culture was incubated at 37 C for 48 hr in a New Brunswick Control Environment shaker with agitation of 200 rpm. The cells were removed from the medium by centrifugation and the supernatant fluid was used as the source of the crude enzyme. Partial purification was achieved by alcohol precipitation, redissolution in water and sequential fractionation at pH 8.6 and 4.3. Further purification was obtained by passing the enzyme preparation through a Sephadex G-200 column (6). This enzyme preparation had a specific activity of 22 μmol butyric acid released per minute per milligram protein.

Enzyme Assay

First 0.5 ml of the enzyme was added to 15

TABLE I

Amount of Added Alcohol Causing 50% Inhibition of Tributyrin Hydrolysis

Alcohol	Log μmol alcohol added causing 50% inhibition of hydrolysis
Propanol	4.0
Butanol	4.0
Pentanol	3.4
Hexanol	2.9
Heptanol	1.6
Octanol	2.5
Decanol	2.7
Dodecanol	3.0

TABLE II

Amount of Added *n*-Alcohol Causing 50% Inhibition of Various Triglyceride Substrates

Alcohol	Log μmol alcohol added causing 50% inhibition of hydrolysis		
	Trimyristin	Tripalmitin	Triolein
Propanol	4.1	4.1	4.1
Butanol	4.0	4.0	4.0
Pentanol	2.9	3.4	3.3
Hexanol	2.8	3.0	3.2
Heptanol	2.1	2.8	2.8
Octanol	2.4	2.8	2.8
Decanol	3.4	3.7	3.7
Dodecanol	3.3	3.7	3.6

ml of a mixture composed of lipid; 1.6% gum accacia; 4.10^{-6} M sodium taurocholate; 0.4 M NaCl and 5 x 10^{-3} M $CaCl_2$ pH 8.0 (7). The concentrations of lipids usually used were tributyrin 10 mM; triolein or triglycerides with long chain fatty acids 34 mM; and tween 80 12.5 mM. As control, the same reaction mixture without lipid was used. The reaction mixture was incubated at 37 C for 10 min and then the reaction was stopped by addition of an equal volume of absolute ethanol. The amount of 0.01 N NaOH required to bring the reaction mixture back to pH 8.0 by electrometric titration in pH stat (Radiometer, Copenhagen) was recorded as the measure of lipase activity.

TABLE III

Amount of Added *n*-Alcohol Causing 50% Inhibition of Tween 80 Hydrolysis

Alcohol	Log μmol alcohol added causing 50% inhibition of hydrolysis
Propanol	4.1
Butanol	3.6
Pentanol	2.7
Hexanol	1.9
Heptanol	1.6
Octanol	1.5
Decanol	1.4
Dodecanol	1.4

Inhibition Studies

The various inhibitors were added to the reaction mixture described previously. Unless otherwise stated, the enzyme was added and the enzymatic activity assessed. ID_{50} is expressed as log μmoles of the inhibitor, which caused 50% inhibition of the reaction.

RESULTS

The inhibitory effect of *n*-alcohols on *Staphylococcus aureus* lipase activity on the substrate glycerol tributyrin is presented in Table I. Maximum inhibition was observed when heptanol was added, whereas an increase or a decrease in the chain length of the *n*-alochol added caused a decrease in the inhibitory effect. When triglycerides with longer chain fatty acids were assayed (Table II) greater amounts of alcohol were needed to inhibit the enzymatic activity, although the pattern of inhibition by the various alcohols is the same as previously described for glycerol tributyrin. The hydrolysis of glycerol tristearin could not be inhibited, even with relatively high concentrations of alcohol. When a compound such as

TABLE IV

Inhibitory Effect of Various Compounds of C_7 on Lipase Activity

C_7 compound	Log μmol compound added causing 50% inhibition of hydrolysis	
	Tributyrin	Tween 80
Heptane	3.8	3.8
1-Heptanol	1.6	1.8
Heptanal	1.5	1.8
2-Heptanol	3.5	3.8
2-Heptanone	2.3	2.8
3-Heptanol	3.7	3.8
3-Heptanone	2.8	3.7
4-Heptanol	3.9	3.6
Cycloheptanol	3.4	3.4

TABLE V

Inhibitory Effect of Various C_5 Alcohols on Activity of *Staphylococcus aureus* Lipase on Tributyrin

Compound	% inhibition by addition of 2.96 log μmol compounds
$CH_3(CH_2)_3CH_2OH$	30
$CH_3(CH_2)_2CH_2OHCH_3$	0
$(CH_3)_2CHCH_2CH_2OH$	24
$(CH_3)_3C\text{-}CH_2OH$	14

Tween 80 (polyoxiethylene sorbitan-monooleate) which is miscible in water and in an organic phase, was used as the substrate (Table III), there was an increase in the inhibitory effect of *n*-alcohols with the elongation of the chain length of the alcohol. However, for alcohols with a chain length of C_7 and higher, almost the same amount of alcohol is needed in order to cause 50% inhibition.

The data presented above suggest that *n*-heptanol is a good inhibitor of lipase activity when triglycerides and Tween 80 are used as substrates. We tested the ability of various compounds of the normal seven carbon chain and cycloheptanol to inhibit enzymatic activity. The data presented in Table IV indicate that the greatest inhibition is obtained by the primary alcohol or aldehyde. As the hydroxyl group is further removed from the end of the chain it loses its inhibitory activity, but when the hydroxyl group is changed to a keto group greater inhibition is obtained. Cycloheptanol has very little inhibitory activity, roughly equaling that of heptane.

When the influence of the carbon chain of the alcohol was tested (Table V), it could be seen that changes in the configuration of the chain influence the inhibitory effect. However a greater loss of the inhibitory activity was obtained when the hydroxyl group was changed (secondary alcohol)

The concentrations of substrate or of enzyme were varied in the presence of 70 μmol heptanol and the effect that this had on the rate of hydrolysis was determined. The results presented in Table VI suggest that addition of substrate diminished the inhibitory activity of the heptanol, whereas no changes could be observed when the concentration of the enzyme was changed (Table VII).

In order to test whether the alcohol is adsorbed onto the enzyme or onto the oil phase of the substrate, heptanol was added to an emulsion of the substrate and mixed for 20 min at 37 C in a shaker water bath. A sample of this mixture was tested for enzymatic activity, while another portion was dialyzed overnight against 20 volumes distilled water and then tested again for enzymatic activity. As control the same amount of heptanol was added to water and tested before and after dialysis for the inhibitory effect. The date presented in Table VIII show that the alcohol is bound to the substrate and not released following dialysis. When the alcohol was added to an enzyme solution and afterwards dialyzed against distilled water, no loss of enzymatic activity was observed.

DISCUSSION

The data presented by us show that in emulsions of triglycerides and different alcohols the maximum inhibitory effect on hydrolysis was observed with heptanol. However, when Tween 80 was used as the substrate, a further increase in the chain length of the alcohol caused an increase in the inhibitory effect similar to that observed by Mattson et al. (4),

TABLE VI

Effect of Varying Amounts of Substrate on Hydrolysis of Glycerol Tributyrin in Presence 70 μmol Heptanol

Substrate μmol	μmol butyric acid released/min: No alcohol	Plus alcohol	Inhibition, %
50	4.0	3.2	20
30	3.8	2.9	23
20	3.2	2.4	25
10	2.8	1.8	36
5	1.9	1.3	30

TABLE VII

Effect of Varying Amounts of Enzyme on Hydrolysis of Glycerol Tributyrin in Presence of 70 μmol Heptanol

Enzyme/μg	μmol butyric acid released/min: No alcohol	Plus alcohol	Inhibition
50	1.98	1.25	37
80	2.82	1.81	36
100	3.45	2.10	39
120	3.69	2.24	39

TABLE VIII

Effect of Dialysis on Hydrolysis of Glycerol Substrate in Presence of 100 μmol Heptanol

	Before dialysis			After dialysis		
	No alcohol	Plus alcohol		No alcohol	Plus alcohol	
Substrate	μmol fatty acid/min		Inhibition, %	μmol fatty acid/min		Inhibition, %
Tributyrin	2.40	0.50	78	2.50	0.65	73
Triolein	1.35	0.47	65	1.25	0.55	56
Control[a]	2.40	0.50	78	2.50	2.40	4

[a]Heptanol was mixed with water and introduced into reaction mixture and tested on the substrate, tributyrin.

who worked on pancreatic lipase with methyl oleate as substrate. The greater inhibition observed with the long carbon chain alcohols is probably due to the fact that these compounds mix better in Tween 80 than in an emulsion of triglycerides. Our data, similar to those of Mitanda et al. (8), who worked on inhibition of lipoxygenase, suggest that the best inhibition is obtained with normal primary alcohols or aldehydes. Removal of the nucleophylic group from the end of the chain or configurational changes in the carbon chain cause a decrease in the inhibitory effect of the compound.

When the solubility in water of the various compounds that inhibit the hydrolysis of triglycerides was analyzed, we found that the best inhibitory effect is obtained with compounds (such as heptanol) that are soluble to a degree of ca. 0.1%. As compounds become more or less soluble than this value, their inhibitory effect decreases at a proportionate rate.

Although there are a number of reports on the possibility of hydrophilic binding between the enzyme and alcohols (9,10) (usually short chain alcohols), our data suggest that the first step in the inhibition is the binding of the alochol to the substrate. This binding is relatively strong and cannot be reversed by dialysis. The fact that heptanol has the greatest inhibitory effect is compatible with the earlier observation of Mathson and Volpenheim (11) that acyl esters of heptanol with various fatty acids served as the best substrates for hydrolysis by pancreatic lipase. It is conceivable, therefore, that the heptanol that is inserted in the droplet of the triglyceride blocks the enzyme from attacking the substrate at interphase. This si also in keeping with the concept that long hydrocarbon chains of fatty acids anchored at the oil-water interphase by the hydrophilic ester linkage are essentially free. Therefore any blocking of this free group causes an inhibition of the enzyme activity.

REFERENCES

1. Benzonana, G., and P. Desnuelle, Biochim. Biophys. Acta. 105:121 (1963).
2. Entressangles, B., and P. Desnuelle, Ibid. 159:285 (1967).
3. Brockerhoff, H., Arch. Biochem. Biophys. 134:366 (1969).
4. Mattson, F.H., R.A. Volpenhein and L. Benjamin, J. Biol. Chem. 245:5335 (1970).
5. Mates, A., Experentia 28:1464 (1972).
6. Vadehra, D.V., and L.G. Hermon, Appl. Microbiol. 15:292 (1967).
7. Vadehra, D.V., and L.G. Hermon, Ibid. 15:480 (1967).
8. Mitsuda, H., K. Yasumoto and A. Yamamoto, Arch. Biochem. Biophys. 118:664 (1967).
9. Miles, J.L., E. Morey, F. Crain, S. Gross, J. San Julan and W.J. Canady, J. Biol. Chem. 237:1319 (1962).
10. Tang, G., Ibid. 240:2810 (1965).
11. Mattson, F.H., and R.A. Volpenhein, J. Lipid Res. 10:271 (1969).

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D-Galactosamine Induced Fatty Liver: II. Analyses of Hepatic Lipid Compositions

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ABSTRACT

Female rats of the Wistar strain received six separate intraperitoneal injections of D-galactosamine (250 mg/kg body wt per single injection). The rats were sacrificed 48 hr after the initial injection and the livers analyzed for their lipid compositions. A marked increase in the levels of total lipids and triglyceride with a pronounced decrease in phospholipid content in the liver was observed. However the percentage of individual phospholipid fractions obtained by thin layer chromatography showed no significant changes after D-galactosamine administration, except for a slight increase in lysophosphatidylcholine. Fatty acid analyses of total lipids and phospholipids demonstrated an increase in the percentage of oleic and linoleic acids, with a decrease in the percentage of arachidonic acid after D-galactosamine administration. In the triglyceride fraction, a slight increase in the percentage of myristic, palmitoleic and linolenic acids was observed.

INTRODUCTION

D-Galactosamine, administered intraperitoneally to rats, produces histological changes similar to those of human viral hepatitis, including acidophilic degeneration of liver cells as well as many acidophilic bodies that are regarded as considerably specific to human viral hepatitis (1). Medline et al. (2) observed histologically that D-galactosamine also produces fatty liver. Koff et al. (3) reported the marked accumulation of hepatic triglyceride in rats administered D-galactosamine. Previous reports (4,5) from this laboratory have shown a significant increase in total lipids of the liver and fatty infiltration of hepatocytes observed under the light and electron microscope. The author and collaborators (6,7) have previously reported that the administration of D-galactosamine produces significant changes in the concentration and fatty acid composition of plasma lipids. The present study was carried out to see whether D-galactosamine administration to rats could modify the lipid composition of hepatic lipids, especially those of phospholipids.

MATERIALS AND METHODS

D-Galactosamine hydrochloride was generously supplied by Research Laboratories of Chugai Pharmaceutical Co. Ltd. Since its specific rotation was $[\alpha]_D^{20}$ +70°, the purity of offered D-galactosamine was calculated as 76.6%. The paper chromatographic analysis revealed that the contaminant of the 23.4% level was chondroitin sulfate.

Seventeen female rats of the Wistar strain, weighing 160-170 g, were used for the experiment; the age of the rats was 8-9 weeks. The animals were fed a standard laboratory chow diet (Oriental Yeast Mfg. Ltd., Japan). They received six separate intraperitoneal injections of a neutral 0.45 M solution of D-galactosamine hydrochloride. The dose was 250 mg/kg body wt per single injection with the first three at 4 hr intervals, followed by a 14 hr pause, and the final three injections at hourly intervals. Control rats received no treatment until sacrifice. All rats were fasted for 15 hr with free access to

TABLE I

Lipid Composition of Liver[a]

Group	No. of animals	Liver wt, g/100g body wt	Total lipids, mg/g liver	Triglyceride, mg/g liver	Phospholipids,[b] mg/g liver
Control	7	3.7 ± 0.2	54.7 ± 2.7	7.9 ± 1.5	35.9 ± 2.1
GalN[c]-treated	7	4.3 ± 0.6 (P<0.05)	126.8 ± 17.0 (P<0.001)	58.6 ± 10.4 (P<0.001)	24.4 ± 0.8 (P<0.001)

[a]Data are expressed as mean and standard deviations.

[b]mg of phospholipids = mg of lipid phosphorus x 25.

[c]D-Galactosamine.

water prior to sacrifice by decapitation 48 hr after the initial injection of D-galactosamine.

The liver was quickly excised and weighed. The liver lipids were extracted according to Folch et al. (8). Total lipids of the liver were determined by weighing.

The triglyceride and phospholipid fractions were obtained by the thin layer chromatographic method of Freeman and West (9) in order to remove the contamination of mono- and diglyceride from the phospholipid fractions. Those triglyceride and phospholipid fractions were subsequently subjected to fatty acid analyses.

Subfractions of phospholipids of the control rats were separated by thin layer chromatography on a Silica Gel H plate (E. Merck, A.G., Germany) according to Skipski et al. (10). In the D-galactosamine treated animals, each plate was developed using petroleum ether-acetone 3:1 v/v as a first developing solvent in order to remove increased hepatic triglyceride. After the solvent front was allowed to run to the top of the plate, the plate was dried under nitrogen flow and rechromatographed using the developing solvent of Skipski et al.

Each lipid fraction was detected under UV light after spraying a 0.2% solution of 2',7'-dichlorofluorescein sodium salt ethanol on the plate. The identification of each spot was carried out using pure reference compound (Applied Science Labs. Inc.).

Gas chromatographic analyses of hepatic total lipids, triglyceride and phospholipids were carried out as described previously (7). Fatty acid levels in hepatic triglyceride and phospholipids were estimated by the method of Horning et al. (11).

To determine the hepatic triglyceride content, 1 g of the liver was homogenized with 10 ml isopropylalcohol and extracted with 40 volumes isopropylalcohol, allowing it to stand overnight. The extract was filtered and the filtrate made up to 50 ml with isopropylalcohol in the control rats, whereas that from D-galactosamine administered animals was made up to 100 ml, 3 ml of which was rediluted to 25 ml with isopropylalcohol. The final solution was subjected to determination of the level of hepatic triglyceride by the method of Fletcher (12). Since the hepatic triglyceride level measured by such a procedure might exhibit somewhat higher value due to the contaminated free glycerol, the hepatic free glycerol level was also measured with each specimen by omitting the step of saponification in Fletcher's method. The value of hepatic triglyceride content was calculated by subtracting the free from the total glycerol.

TABLE II

Percentage of Lipid Phosphorus in Individual Phospholipid Fractions of Liver[a]

Group	No. of animals	Phospholipid fractions[b]						
		Origin	LPC	SM	PC	PS+PI	PE	Front
Control	5	0.2 ± 0.2	1.3 ± 0.3	3.5 ± 0.8	49.1 ± 2.7	11.5 ± 1.2	27.2 ± 1.9	7.2 ± 0.1
GalN[c]-treated	6	0.2 ± 0.1	1.8 ± 0.4 ($P<0.05$)	4.0 ± 0.7	48.2 ± 1.0	11.7 ± 0.5	26.9 ± 1.7	7.4 ± 0.7

[a]Data are expressed as mean and standard deviations.

[b]Abbreviations: LPC, lysophosphatidylcholine; SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine.

[c]D-Galactosamine.

Lipid phosphorus was measured by the method of Bartlett (13). The amount of phospholipids was calculated by multiplying the amount of lipid phosphorus by 25.

RESULTS

Lipid Composition of Liver (Table I)

The liver wet weight per 100 g body weight increased significantly in D-galactosamine treated animals. The D-galactosamine administration produced a marked elevation in the levels of total lipids and triglyceride, with a pronounced decrease in phospholipid content in the liver.

Individual Fractions of Hepatic Phospholipids (Table II)

The individual phospholipid fractions obtained by thin layer chromatography are as follows: origin (nonlipid phosphorus), lysophosphatidylcholine, sphingomyelin, phosphatidylcholine, combined fraction of phosphatidylserine and phosphatidylinositol, phosphatidylethanolamine and front (cardiolipin, triglyceride, etc.).

The liver of D-galactosamine treated animals contained a lesser amount of phospholipids, whereas the percentage of recovered phosphorus of each subfraction showed no significant changes, except for a slight increase in lysophosphatidylcholine.

Fatty Acid Composition of Hepatic Lipids (Table III)

In the fatty acid composition of hepatic total lipids, an increase in the percentage of palmitoleic, oleic and linoleic acids and a decrease in the percentage of arachidonic acid were noted after D-galactosamine administration. In the phospholipid fraction, an increase in the percentage of oleic and linoleic acids and a decrease in arachidonic and docosahexaenoic acids were observed. However almost no significant changes were produced by D-galactosamine administration in the fatty acid composition of triglyceride fraction, except for a slight increase in the percentage of myristic, palmitoleic and linolenic acids. Thus the changes of fatty acid composition expressed as per cent were most remarkable in total lipids.

Estimated Amount of Fatty Acids (Table IV)

In the triglyceride fraction, the absolute levels of fatty acids changed strikingly after D-galactosamine administration, whereas only slight changes were observed in the fatty acid composition when expressed as per cent. The concentration of stearic and arachidonic acids decreased in the phospholipid fraction after D-galactosamine administration.

TABLE III

Fatty Acid Composition of Hepatic Lipids[a]

Fatty acids	Total lipids			Triglyceride			Phospholipids		
	Control, 5 rats, %	GalN[a]-treated, 10 rats, %		Control, 5 rats, %	GalN-treated, 10 rats, %		Control, 5 rats, %	GalN-treated, 10 rats, %	
14:0	0.5 ± 0.6	0.9 ± 0.2		0.5 ± 0.2	1.1 ± 0.3	(P<0.02)	Trace	Trace	
16:0	23.0 ± 3.1	24.6 ± 1.9		28.7 ± 2.5	26.3 ± 1.4		16.0 ± 0.6	19.4 ± 3.5	
16:1	3.0 ± 1.1	6.4 ± 1.1	(P<0.001)	5.5 ± 1.3	8.0 ± 0.3	(P<0.02)	0.8 ± 0.4	0.4 ± 0.0	
17:0	---	---		---	---		Trace	0.1 ± 0.0	(P<0.001)
18:0	21.2 ± 2.6	8.3 ± 2.1	(P<0.001)	1.8 ± 0.3	1.9 ± 0.3		34.3 ± 2.5	33.2 ± 6.3	
18:1	18.2 ± 1.1	29.8 ± 0.6	(P<0.001)	35.3 ± 0.6	36.6 ± 3.3		6.2 ± 1.3	7.9 ± 1.2	(P<0.05)
18:2	18.3 ± 1.0	24.4 ± 2.0	(P<0.001)	26.7 ± 3.4	24.6 ± 2.6		11.9 ± 2.5	17.3 ± 1.8	(P<0.001)
18:3	Trace	0.9 ± 0.4	(P<0.01)	Trace	1.1 ± 0.2	(P<0.001)	Trace	Trace	
20:3	Trace	Trace		---	---		Trace	0.2 ± 0.3	
20:4	15.7 ± 3.5	4.8 ± 0.6	(P<0.01)	1.6 ± 1.0	0.6 ± 0.5		24.2 ± 3.8	18.0 ± 3.2	(P<0.01)
22:6	Trace	Trace		---	---		6.6 ± 1.1	3.6 ± 1.5	(P<0.001)

[a]Data are expressed as mean and standard deviations.
[b]D-Galactosamine.

TABLE IV

Estimated Amount of Fatty Acids[a]

Fatty acids	Control, mg/g liver			GalN[b]-treated, mg/g liver		
	Triglyceride	Phospholipids	Total	Triglyceride	Phospholipids	Total
14:0	0.0	0.0	0.0	0.7	0.0	0.7
16:0	2.3	3.7	6.0	15.4	3.1	18.5
16:1	0.4	0.2	0.6	4.7	0.1	4.8
17:0	—	—	—	—	0.0	0.0
18:0	0.1	8.0	8.1	1.1	5.3	6.4
18:1	2.8	1.4	4.2	21.5	1.3	22.8
18:2	2.1	2.8	4.9	14.4	2.7	17.1
18:3	—	—	—	0.7	—	0.7
20:3	—	—	—	—	0.0	0.0
20:4	0.1	5.6	5.7	0.4	2.9	3.3
22:6	—	1.5	1.5	—	0.6	0.6
Total	7.8	23.2	31.0	58.9	16.0	74.9

[a]Values are calculated from the data in Tables I and III. Mean values are used for calculation. mg of triglyceride x 1 = mg of triglyceride fatty acid; mg of phospholipids x 0.65 = mg of phospholipid fatty acid.

[b]D-Galactosamine.

DISCUSSION

A number of agents such as carbon tetrachloride, ethionine, orotic acid and puromycin have been reported to produce fatty liver. It is well recognized that the mechanism of fatty liver production by these agents is impaired synthesis of lipoprotein or disturbance in release of lipoprotein from the liver.

D-Galactosamine induces histological as well as biochemical changes similar to those of human viral hepatitis but also produces fatty liver (1-6). Koff et al. (3,14) observed marked accumulation of hepatic triglyceride, impairment of the incorporation of ^{14}C-leucine into microsomal protein and reduced posttriton hypertriglyceridemia in rats administered D-galactosamine. They concluded that the cause of D-galactosamine induced fatty liver was inhibition of synthesis of the protein moiety of lipoprotein.

The author's present data show that phospholipid content in the liver is reduced, but the percentage of individual phospholipid fractions remains almost unchanged after D-galactosamine administration. It is well recognized that phospholipids play an important role in lipoprotein formation (15,16). It has also been reported that the decrease in hepatic phospholipid content impairs fatty acid oxidation (17,18). Thus it can conceivably be said that the overall decrease in hepatic phospholipid concentration may play at least a partial role in the production of D-galactosamine induced fatty liver.

It has been reported that carbon tetrachloride reduces hepatic phospholipid content (19,20). The important role of phospholipids on fatty liver production in carbon tetrachloride intoxication has been pointed out and studied by several investigators (19,21-24). Sgoutas (23) reported that the administration of carbon tetrachloride slightly increased the levels of the combined fraction of sphingomyelin and lysophosphatidylcholine as well as phosphatidylinositol, decreased the levels of phosphatidylserine and phosphatidylethanolamine, and produced no change in cardiolipin and phosphatidylcholine content in rat liver. Because the author's present data show the overall decrease in phospholipid subfractions except for a slight increase in the level of lysophosphatidylcholine after D-galactosamine administration, it is suggested that there are some qualitative differences in the impaired phospholipid metabolism between D-galactosamine and carbon tetrachloride induced fatty liver.

In fatty acid analyses of hepatic lipids, major findings were an increase in the percentage of oleic and linoleic acids, and a decrease in that of arachidonic acid. These data are in agreement with those reported in carbon tetrachloride (11) and ethionine (11,25) induced fatty liver. D-Galactosamine induced alterations in fatty acid composition of hepatic lipids were similar to those of plasma lipids which were reported previously (7).

Since the triglycerides and phospholipids presumably constitute most of the fatty acid-containing hepatic lipids, the fatty acid changes in hepatic total lipids may reflect changes in those two fractions. As shown in Table IV, it appears likely that an increase in the percentage of palmitoleic, oleic and linoleic acids of hepatic total lipids reflects an increase of those

fatty acids in the triglyceride fraction. Those fatty acids are possibly mobilized from the adipose tissue, since they are the main fatty acids of adipose tissue (25) and the level of plasma free fatty acids increases after D-galactosamine administration (7). Similarly, a decrease in the percentage of stearic and arachidonic acids may reflect a decrease of those fatty acids in the phospholipid fraction. A decrease in the concentration of arachidonic acid is possibly due to an impairment of chain elongation and desaturation of linoleic acid.

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REFERENCES

1. Keppler, D., R. Lesch, W. Reutter and K. Decker, Exp. Mol. Pathol. 9:279 (1968).
2. Medline, A., F. Schaffner and H. Popper, Ibid. 12:201 (1970).
3. Koff, R.S., G. Gordon and S.M. Sabesin, Proc. Soc. Exp. Biol. Med. 137:696 (1971).
4. Okazaki, I., K. Kamo, E. O, M. Oda, K. Kamegaya, M. Tsuchiya and K. Sambe, Acta Hepatologica Japonica 12:288 (1971).
5. Sambe, K., M. Tsuchiya, K. Kamegaya, M. Oda and I. Okazaki, Ibid. 13:85 (1972).
6. Morita, A., K. Suzuki, T. Morishita, H. Asakura, M. Oda, K. Kamegaya, M. Tsuchiya and K. Sambe, Ibid. 13:186 (1972).
7. Morita, A., Biochem. Exp. Biol., In press.
8. Folch, J., M. Lees and G.H. Sloane-Stanley, J. Biol. Chem. 226:497 (1957).
9. Freeman, C.P., and D. West, J. Lipid Res. 7:324 (1966).
10. Skipski, V.P., R.F. Peterson and M. Barclay, Biochem. J. 90:374 (1964).
11. Horning, M.G., M.J. Earle and H.M. Maling, Biochim. Biophys. Acta 56:175 (1962).
12. Fletcher, M.H., Clin. Chim. Acta 22:393 (1968).
13. Bartlett, G.R., J. Biol. Chem. 234:466 (1959).
14. Koff, R.S., J.J. Fitts and S.M. Sabesin, Proc. Soc. Exp. Biol. Med. 138:89 (1971).
15. Fleischer, B., and S. Fleischer, Biochim. Biophys. Acta 147:566 (1967).
16. Sodhi, H.S., and R.G. Gould, J. Biol. Chem. 242:1205 (1967).
17. Rodbell, M., and D.J. Hanahan, Biol. Chem. 214:595 (1955).
18. Artom, C., Amer. J. Clin. Nutr. 6:221 (1958).
19. Rossi, F., and M. Zatti, Brit. J. Exp. Pathol. 44:131 (1963).
20. Aiyar, A.S., P. Fatterpaker and A. Sreenivasan, Biochem. J. 90:558 (1964).
21. Zatti, M., F. Rossi and G. Zoppi, Experientia 21:215 (1965).
22. Halbreich, A., and J. Mager, Biochim. Biophys. Acta 187:584 (1969).
23. Sgoutas, D.S., Metabolism 16:382 (1967).
24. Shimizu, Y., J. Lipid Res. 10:479 (1969).
25. Arvidson, G., and T. Olivecrona, Acta Physiol. Scand. 55:303 (1962).

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Mass Spectral Analysis of Eleven Analogs of Vitamin A[1]

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ABSTRACT

The low resolution mass spectra of 5,6-monoepoxyretinoic acid, 5,8-monoepoxyretinoic acid, 5,6-monoepoxyretinyl acetate, 5,8-monoepoxyretinyl acetate, 5,8-monoepoxyretinal, trimethylsilyl-5,6-monoepoxyretinoate, trimethylsilyl-5,8-monoepoxyretinoate, methyl-5,6-monoepoxyretinoate, methyl-5,8-monoepoxyretinoate, β-C_{19}-retinal and β-C_{19}-retinyl acetate—analogs of vitamin A—are presented. Characteristic features of the spectra are discussed. In addition, high resolution mass measurements are presented for six fragment ions of all-*trans*-retinoic acid and eleven fragment ions of methyl-5,6-monoepoxyretinoate.

INTRODUCTION

A number of workers have reported the isolation of metabolites of retinoic acid (1-11); however these compounds have been only partially characterized and only two, 13-*cis*-retinoic acid (9) and retinoyl-β-glucuronide (7,10,11), have been identified. In both cases identification involved chromatographic separation and comparison to samples of known structure. Furthermore the decarboxylation of retinoic acid in vivo (6) and in vitro (1,6,7) has been reported, but the products have not been identified. Although Vecchi et al. (12) and Lin et al. (13) have published mass spectra of some vitamin A compounds, interpretation of mass spectra of vitamin A metabolites of unknown structure would be aided by spectra of other structural analogs of vitamin A. This paper reports the mass spectra of 5,6-monoepoxyretinoic acid, 5,8-monoepoxyretinoic acid, 5,6-monoepoxyretinyl acetate, 5,8-monoepoxyretinyl acetate, 5,8-monoepoxyretinal, trimethylsilyl-5,6-monoepoxyretinoate, trimethylsilyl-5,8-monoepoxyretinoate, methyl-5,6-monoepoxyretinoate, methyl-5,8-monoepoxyretinoate, β-C_{19}-retinal, and β-C_{19}-retinyl acetate.

EXPERIMENTAL PROCEDURES

Materials and Methods

The compounds investigated were obtained from the following sources. The 5,6-mono-

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[2]Present address: Oklahoma Medical Research Foundation, 825 NE 13th, Oklahoma City, Okla. 73106. These data were taken in part from a thesis submitted by the senior author in partial fulfillment of the requirement for the M.Sc., Oklahoma State University, July 1972.

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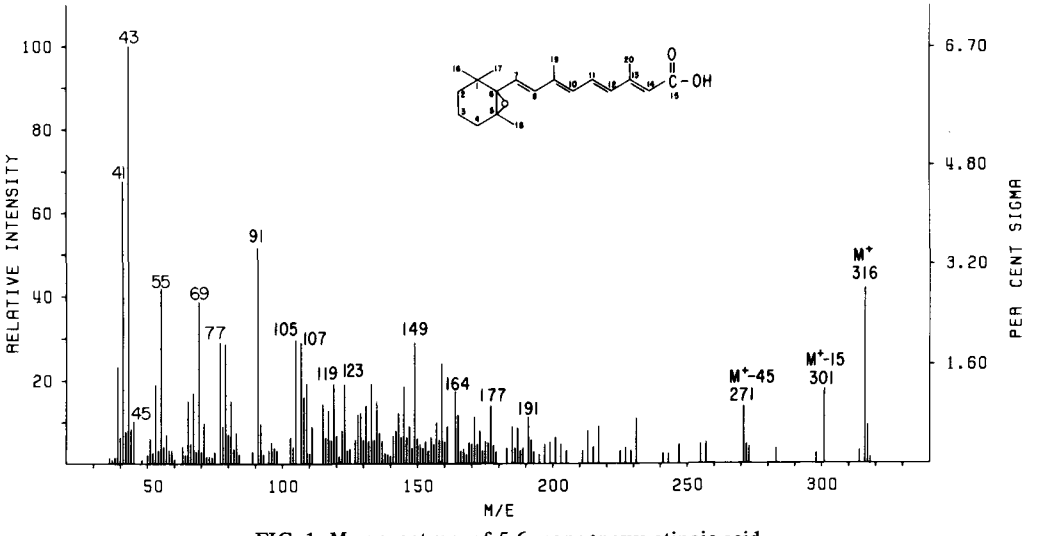

FIG. 1. Mass spectrum of 5,6-monoepoxyretinoic acid.

TABLE I

Fragment Ions of Vitamin A Analogs

Compound	Molecular ion	Interpretive fragment ions		Most abundant fragment ions in decreasing order of intensity
5,6-Monoepoxyretinoic acid	316	301	$(M\text{-}CH_3[15])^+$	43[a], 41, 91, 316, 55, 69, 105, 107, 77, 149
		271	$(M\text{-}COOH[45])^+$	
		45	$[COOH]^+$	
5,8-Monoepoxyretinoic acid	316	301	$(M\text{-}CH_3[15])^+$	43, 149, 41, 91, 95, 316, 164, 55, 107, 93
		271	$(M\text{-}COOH[45])^+$	
		45	$(COOH)^+$	
5,6-Monoepoxyretinyl acetate	344	329	$(M\text{-}CH_3[15])^+$	43, 284, 129, 149, 165, 95, 41, 55, 69, 286, 119, 106, 107
		284	$(M\text{-}CH_3COOH[60])^+$	
		271	$(M\text{-}CH_2OOCCH_3[73])^+$	
		60	$(CH_3COOH)^+$	
5,8-Monoepoxyretinyl acetate	344	329	$(M\text{-}CH_3[15])^+$	43, 284, 165, 149, 269, 95, 121, 344, 285, 271
		284	$(M\text{-}CH_3COOH[60])^+$	
		271	$(M\text{-}CH_2OOCCH_3[73])^+$	
		60	$(CH_3COOH)^+$	
5,8-Monoepoxyretinal	298	283	$(M\text{-}CH_3[15])^+$	149, 191, 43, 95, 269, 177, 41, 123, 135, 107
		269	$(M\text{-}CHO[29])^+$	
		29	$(CHO)^+$	
Trimethylsilyl-5,6-monoepoxyretinoate	388	373	$(M\text{-}CH_3[15])^+$	73, 43, 75, 91, 149, 388, 271, 183, 69, 107, 55
		271	$(M\text{-}COOSi[CH_3]_3[177])^+$	
Trimethylsilyl-5,8-monoepoxyretinoate	388	373	$(M\text{-}CH_3[15])^+$	73, 43, 41, 149, 271, 164, 388, 75, 53, 69
		271	$(M\text{-}COOSi[CH_3]_3[117])^+$	
Methyl-5,6-monoepoxyretinoate	330	315	$(M\text{-}CH_3[15])^+$	330, 149, 271, 43, 315, 91, 69, 107, 135, 217
		271	$(M\text{-}COOCH_3[59])^+$	
		59	$(COOCH_3)^+$	
Methyl-5,8, monoepoxyretinoate	330	315	$(M\text{-}CH_3[15])^+$	330, 149, 271, 43, 315, 107, 164, 95, 91, 177, 135
		271	$(M\text{-}COOCH_3[59])^+$	
		59	$(COOCH_3)^+$	
β-C_{19}-retinal	272	257	$(M\text{-}CH_3[15])^+$	95, 272, 121, 41, 105, 107, 55, 119, 91, 136
		243	$(M\text{-}CHO[29])^+$	
		29	$[CHO]^+$	
β-C_{19}-retinyl acetate	316	301	$(M\text{-}CH_3[15])^+$	43, 41, 120, 105, 107, 119, 91, 55, 93, 79
		256	$(M\text{-}CH_3COOH[60])^+$	
		243	$(M\text{-}CH_2OOCCH_3[73])^+$	
		60	$(CH_3COOH)^+$	

[a]First value is base peak.

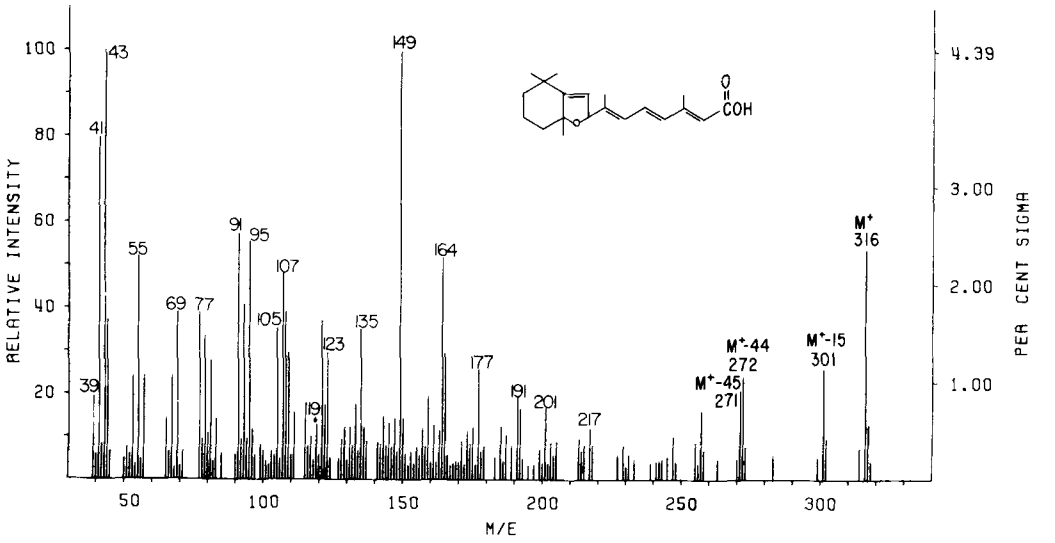

FIG. 2. Mass spectrum of 5,8-monoepoxyretinoic acid.

epoxy- and 5,8-monoepoxy-vitamin A compounds were prepared according to John et al. (14). The β-C_{19}-retinal and all-*trans*-retinoic acid were provided by R.H. Bunnell of Hoffmann-LaRoche, Inc. (Nutley, N.J.). For the preparation of the β-C_{19}-analogs, β-C_{19}-retinal was reduced with $LiAlH_4$ in anhydrous diethyl ether at 4 C. The alcohol was acetylated with acetyl chloride in pyridine to form β-C_{19}-retinyl acetate. The purity of these compounds was established by thin layer chromatography (TLC) on silicic acid, column chromatography on alumina and gas liquid chromatography (GLC) just prior to mass spectral analysis; each compound appeared as one peak on GLC or a single compound on TLC.

Low resolution mass spectra were obtained on ca. 1 μg of compound using the direct inlet probe of the LKB-9000P combination mass spectrometer-gas chromatograph. (Details of instrument design and modifications are reported by G.R. Waller, Proc. Okla. Acad. Sci. 47:271 [1968] and are available upon request.) The probe temperature was varied from ambient to 50 C and the ion source was 310 C. Spectra were taken at 70 eV using an accelerating voltage of 3.5 kV and a trap current of 60 μA.

High resolution mass spectra were obtained on a CEC 21-110-B mass spectrometer. Spectra were taken at 70 eV with a trap current of 75 μA. The direct inlet and source temperatures

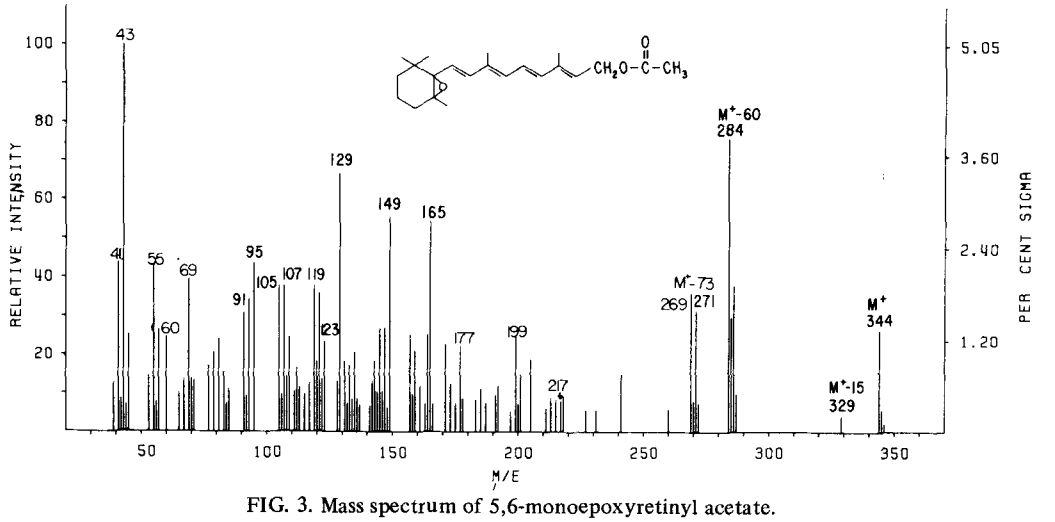

FIG. 3. Mass spectrum of 5,6-monoepoxyretinyl acetate.

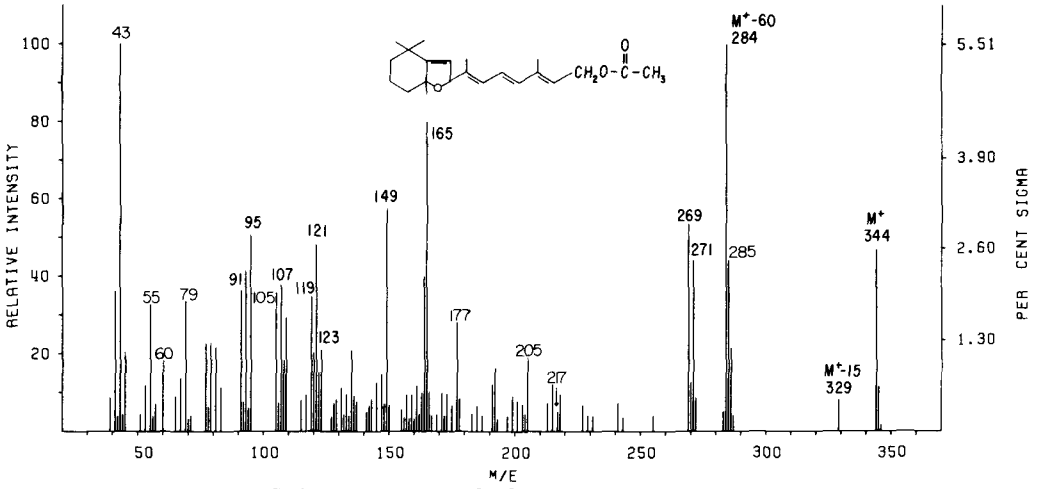

FIG. 4. Mass spectrum of 5,8-monoepoxyretinyl acetate.

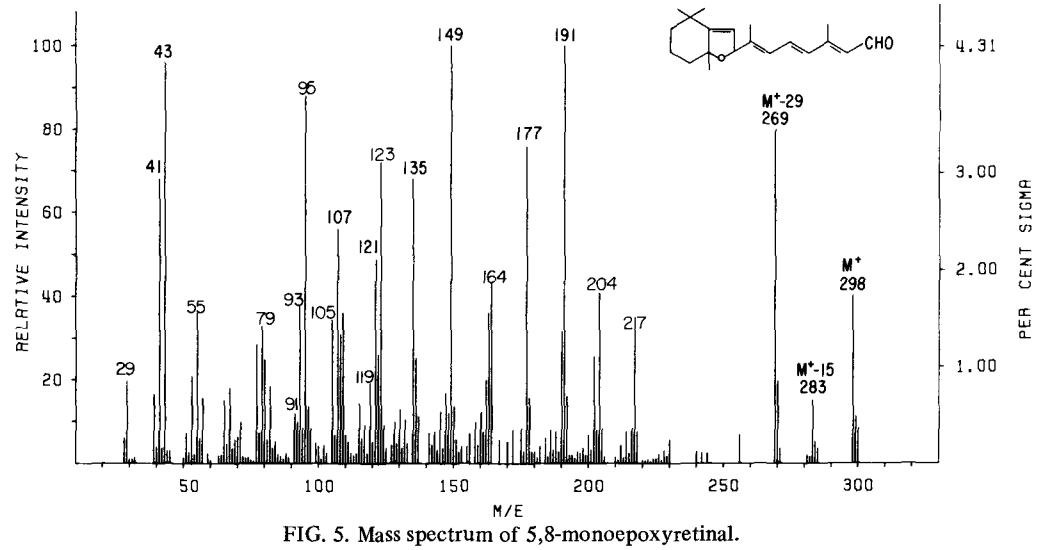

FIG. 5. Mass spectrum of 5,8-monoepoxyretinal.

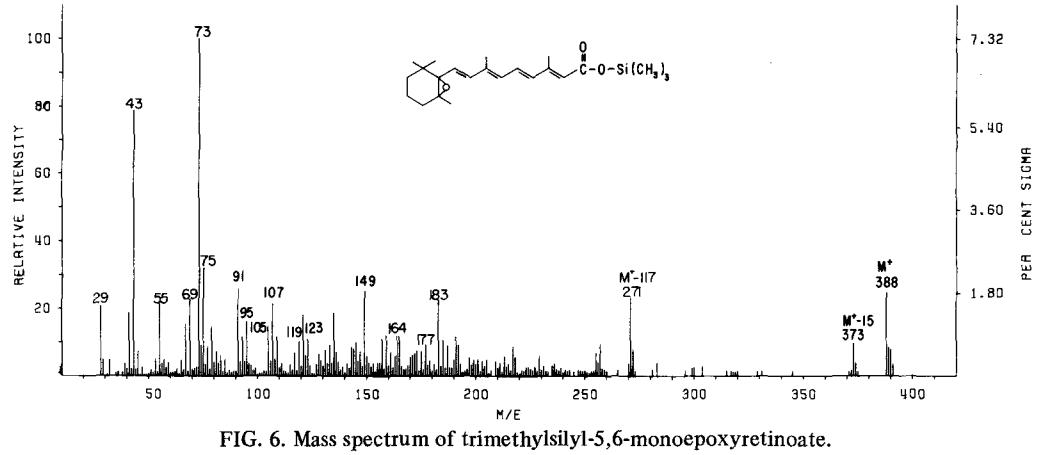

FIG. 6. Mass spectrum of trimethylsilyl-5,6-monoepoxyretinoate.

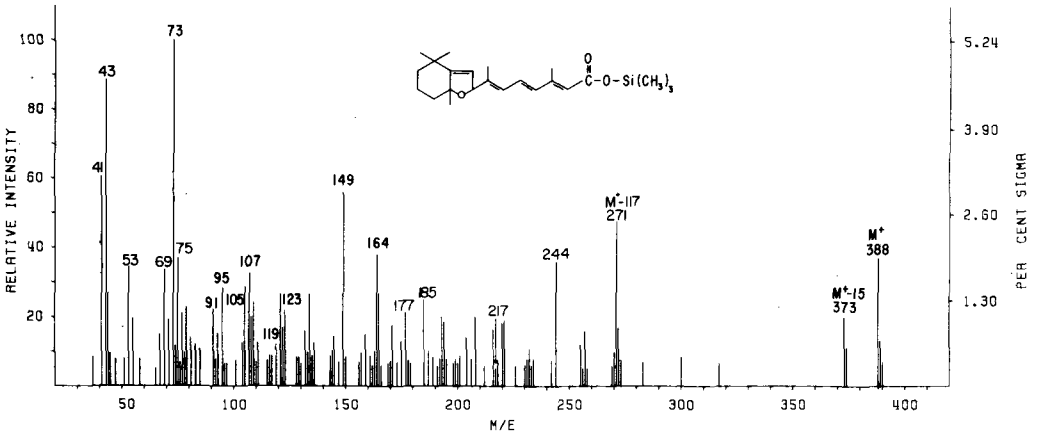

FIG. 7. Mass spectrum of trimethylsilyl-5,8-monoepoxyretinoate.

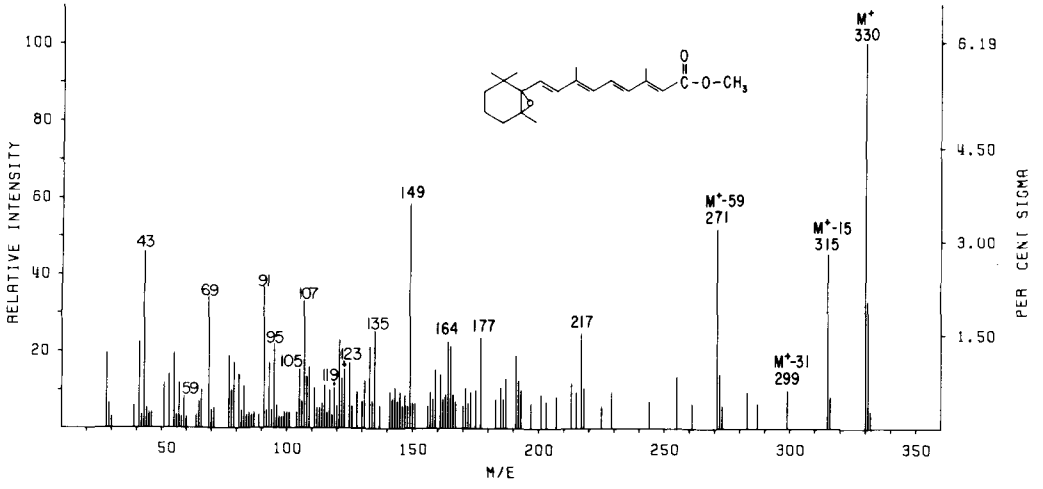

FIG. 8. Mass spectrum of methyl-5,6-monoepoxyretinoate.

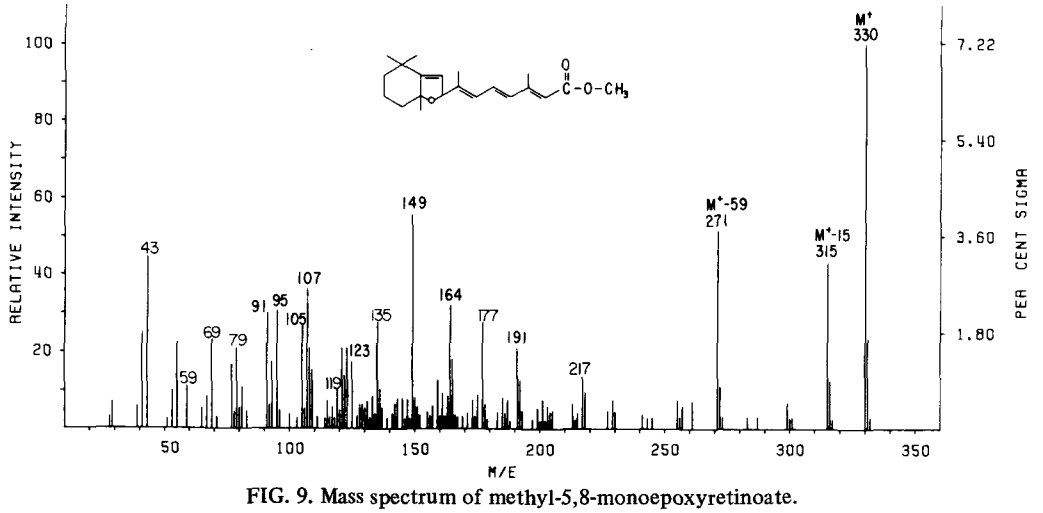

FIG. 9. Mass spectrum of methyl-5,8-monoepoxyretinoate.

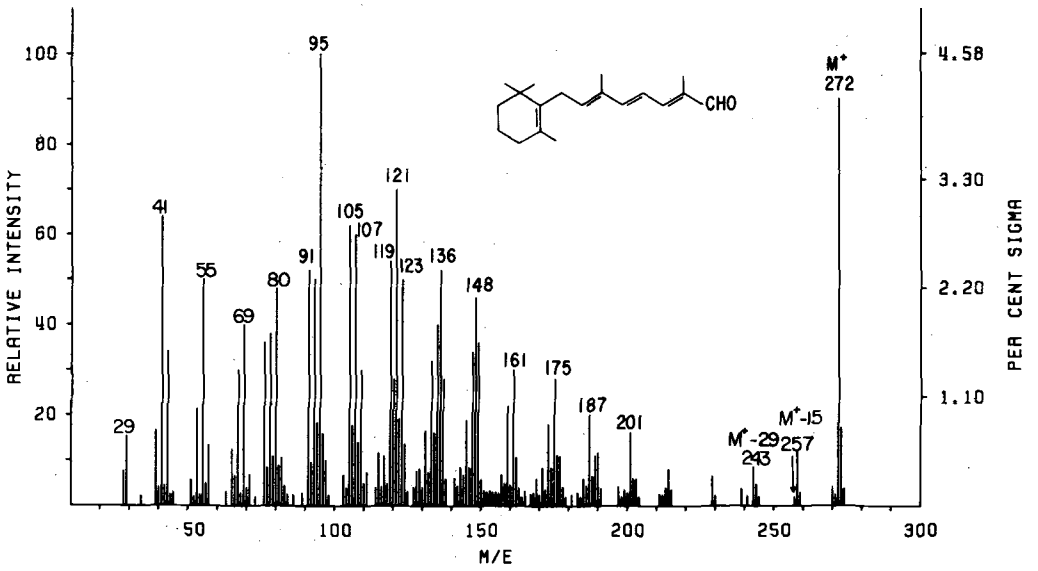

FIG. 10. Mass spectrum of β-C_{19}-retinal.

were 120 C and 150 C, respectively. Mass measurements were accurate to within three millimass units.

RESULTS AND DISCUSSION

The mass spectra and structures of 5,6-monoepoxyretinoic acid, 5,8-monoepoxyretinoic acid, 5,6-monoepoxyretinyl acetate, 5,8-monoepoxyretinyl acetate, 5,8-monoepoxyretinal, trimethylsilyl-5,6-monoepoxyretinoate, trimethylsilyl-5,8-monoepoxyretinoate, methyl-5,6-monoepoxyretinoate, methyl-5,8-monoepoxyretinoate, β-C_{19}-retinal and β-C_{19}-retinyl acetate are shown in Figures 1 through 11, respectively. The 10 most intense peaks of each spectrum and other important interpretive peaks are shown in Table I.

Examination of the above mass spectra, as well as those previously published (12,13), shows a pattern of fragmentation common to all vitamin A analogs studied. The nine fragment ions *m/e* 123, 119, 107, 105, 95, 93, 91, 43 and 41 are present in each spectrum. High resolution mass spectra analysis of six of these fragment ions (Table II) from all-*trans*-retinoic acid (13) and methyl-5,6-monoepoxyretinoate (Fig. 8) revealed that each of the ions was a

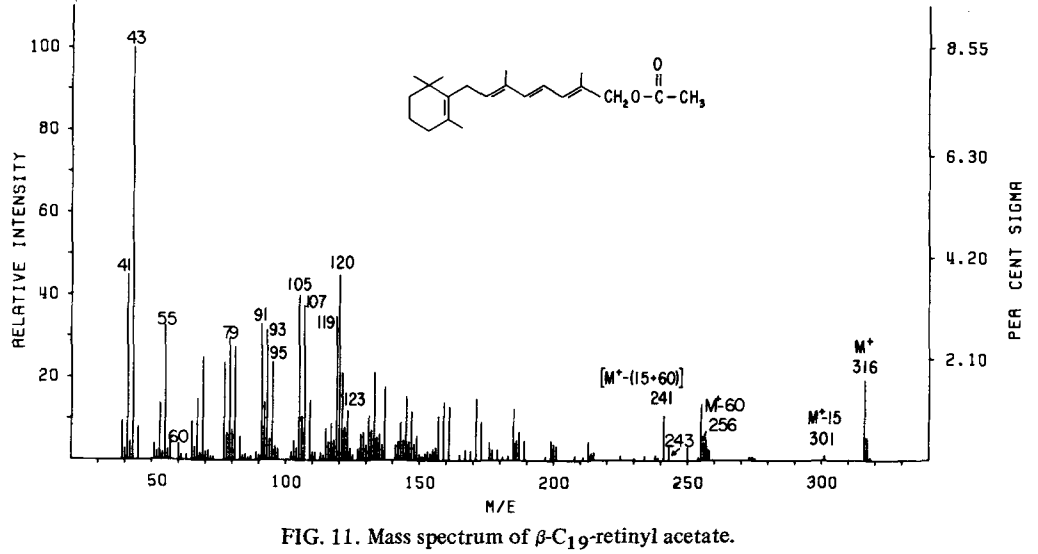

FIG. 11. Mass spectrum of β-C_{19}-retinyl acetate.

TABLE II

High Resolution Measurement of Selected Ions of Methyl-5,6-Monoepoxyretinoate and Retinoic Acid

m/e	Fragment ion composition	Theoretical mass	Observed mass	
			Methyl-5,6-monoepoxyretinoate	Retinoic acid
91	C_7H_7	91.055	91.053	91.055
93	C_7H_9	93.070	93.070	93.070
105	C_8H_9	105.070	105.070	105.071
107	C_8H_{11}	107.086	107.087	107.085
119	C_9H_{11}	119.086	119.089	119.086
123	C_9H_{15}	123.117	123.116	123.117
149	$C_{10}H_{13}O$	149.097	149.098	
164	$C_{11}H_{16}O$	164.120	164.123	
165	$C_{11}H_{17}O$	165.129	165.129	
177	$C_{12}H_{17}O$	177.128	177.129	
217	$C_{15}H_{21}O$	217.159	217.161	
271	$C_{19}H_{27}O$	271.206	271.210	
330	$C_{21}H_{30}O_3$	330.220	330.218	

hydrocarbon and, by analogy, that these ions are probably hydrocarbons in the other compounds studied. The absence of these characteristic peaks from the spectrum of an unknown sample would probably rule out the possibility of a vitamin A analog. However one must examine the entire fragmentation pattern of compounds possessing these ions, since the individual ions also occur in the mass spectra of some steroids and higher terpenes, i.e., cholestane (15).

Comparison of the spectra from the series of 5,6-monoepoxides (Fig. 1, 3, 6 and 8) with their respective analogs from the 5,8-monoepoxides (Fig. 2, 4, 7 and 9) shows very little difference either in the observed pattern of fragmentation or in the relative intensities of the various ions. These results suggest that one or both of two possible processes is occurring. Since in each case the 5,8-isomer was obtained by acid-catalyzed rearrangement of the 5,6-isomer, a thermal rearrangement on the direct inlet probe of the 5,6 to 5,8 isomer is possible. An alternative possibility is that rearrangement occurs upon electron impact, giving rise to a common parent ion for both isomers. The mass spectra of the epoxy analogs of vitamin A (Figs. 1-9) are readily distinguished from those of other vitamin A compounds (13) by the series of ions *m/e* 271, 217, 177, 165, 164 and 149. High resolution mass spectra were obtained for these ions in the spectrum of methyl-5,6-monoepoxyretinoate (Fig. 8) and were found to contain oxygen (Table II).

In addition to the series of ions common to all vitamin A spectra, cleavage of the bond alpha to the terminal functional group was observed in each case. Thus the identity of terminal functional groups may be established by this characteristic cleavage. Each acid (Figs. 1-2) lost COOH to give an $(M-45)^+$ ion; acetates (Figs. 3, 4 and 11) lost CH_2-$COOCH_3$ to give an $(M-73)^+$ ion; aldehydes (Figs. 5 and 10) lost CHO to give $(M-29)^+$; trimethylsilyl esters (Figs. 6-7) lost $COOSi(CH_3)_3$ to give $(M-117)^+$; and methyl esters (Figs. 8-9) lost $COOCH_3$ to give $(M-59)^+$. All compounds studied also exhibited the characteristic loss of a methyl group $(M-15)^+$.

The C_{20}-acetates (Figs. 3-4) lost acetic acid, giving rise to a characteristic $(M-60)^+$, as well as cleavage alpha to the functional group to give $(M-73)^+$. The fragmentations of the β-C_{19}-acetate (Fig. 11) was quite different in intensity from the C_{20}-monoepoxyretinyl acetates (Figs. 3-4); the fragmentation to give $(M-73)^+$ was very small, as was the process giving $(M-60)^+$. The characteristic loss of a methyl group to give $(M-15)^+$ was less intense than in any of the C_{20}-analogs. The intensity of the ion resulting from the loss of a methyl group was also much smaller in the case of β-C_{19}-retinal than in the C_{20}-analogs. These differences in fragmentation may be due to the difference in the stereochemistry of the compounds brought about by the shortening of the side chain and in the loss of conjugation with the ring. These results are in complete agreement with the observations previously reported by Vecchi et al. (12) and by Lin et al. (13) for other vitamin A compounds.

The present report establishes the following characteristic features of the mass spectra of vitamin A analogs: (a) hydrocarbon fragments of *m/e* 123, 119, 107, 105, 95, 93 and 91; (b) cleavage of the bond alpha to a terminal functional group, with a corresponding low mass fragment; and (c) loss of one methyl group before loss of the functional group, giving an additional confirmation of vitamin A-like structure. The important question of the

origin of the hydrocarbon fragments cannot be resolved in the absence of isotopically labeled analogs. These fragments may arise from either the side chain or the ring or both. For example the ion *m/e* 91 occurs with a high relative intensity in the mass spectrum of carotenoids that lack cyclic end groups; it is the base peak in the spectrum of lycoxanthin (16). These data, however, do not establish the origin of these fragments since β-ionone lacks the side chain but gives rise to the complete series *m/e* 91-*m/e* 123 in its spectrum (17). Whether these fragments arise from the side chain, ring, or both, they remain characteristic of a vitamin A carbon skeleton and are thus of potential value in the assignment of structure to metabolites. There is some evidence that the fragmentation of epoxy derivatives of vitamin A compounds may differ from that of β-ionone. Thomas and Willham (17) demonstrated that the methyl group lost from β-ionone is the one at C-5 and not one of the *gem*-dimethyl groups. In the spectra of the oxygen-substituted vitamin A analogs, the loss of a methyl group and the presence of an ion of *m/e* 165 was observed. This ion also has been observed in the mass spectra of the analogous carotenoid and furanoid epoxides by Baldas et al. (18). According to the proposed fragmentation for the carotenoids, this ion retains both the methyl group at C-5 and the *gem*-dimethyl groups.

In the case of cleavage alpha to a terminal functional group,no ambiguity arises and this particular fragmentation feature should allow a definitive determination of the functionality at the chain terminus. The occurence of all features noted in an unknown metabolite should allow for structural assignments with reasonable confidence.

ACKNOWLEDGMENTS

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REFERENCES

1. Nelson, E.C., M. Mayberry, R. Reid and K.V. John, Biochem. J. 121:731 (1971).
2. Nelson, E.C., B.A. Dehority and H.S. Teague, Anal. Biochem. 11:418 (1965).
3. Krishnamurthy, S., J.G. Bieri and E.L. Andrews, J. Nutr. 79:503 (1963).
4. Yagishita, K., P.R. Sundaresan and G. Wolf, Nature 203:410 (1964).
5. Zile, M., and H.F. DeLuca, Biochem. J. 97:180 (1965).
6. Roberts, A.B., and H.F. DeLuca, J. Lipid Res. 9:501 (1968).
7. Lippel, K., D.R. Manyan and A. Llewellyn, Arch. Biochem. Biophys. 139:421 (1970).
8. Wolf, G., J.G. Bergan and P.R. Sundaresan, Biochim. Biophys. Acta 69:524 (1963).
9. Zile, M., R.J. Emerick and H.F. DeLuca, Ibid. 141:639 (1967).
10. Olson, J.A., Amer. J. Clin. Nutr. 22:953 (1969).
11. Lehman, D., Ph.D. dissertation, Oklahoma State University, 1971.
12. Vecchi, M., W. Vetter, W. Walther, S.F. Jermstad and G.W. Schutt, Helv. Chimica Acta 50:1243 (1967).
13. Lin, R.L., G.R. Waller, E.D. Mitchell, K.S. Yang and E.C. Nelson, Anal. Biochem. 35:435 (1970).
14. John K.V., M.R. Lakshmanan and H.R. Cama, Biochem. J. 103:539 (1967).
15. Biemann, K., "Mass Spectrometry: Organic Chemical Applications," McGraw-Hill Book Company, Inc., New York, 1962, p. 339.
16. Enzell, C.R., R.A. Appleton and I. Wahlberg, in "Biochemical Applications of Mass Spectrometry," Edited by G.R. Waller, Wiley Interscience, New York, 1972, p. 375.
17. Thomas, A.F., and B. Willhalm, Tetrahedron Lett. 50:5129 (1967).
18. Baldas, J., Q.N. Porter, L. Cholnoky, J. Szaboles and B.C.L. Weedon, Chem. Comm. 23:852 (1966).

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Biological Effects of Autoxidized Safflower Oils[1]

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ABSTRACT

Studies on the biological effects of autoxidized safflower oil are reported. Autoxidized safflower oil (peroxide value 465 me/kg) was administered daily by intubation in doses of 1 ml to weanling male rats of the Sprague-Dawley strain. Groups of rats were killed at 3 days, 3 weeks and 3 months after starting the daily doses. The growth curves, weights of organs, light and electron microscopic examinations, chemical analysis of blood and liver, and the in vitro conversion of 1-^{14}C-sodium acetate to CO_2, cholesterol and fatty acids in liver slices were studied. Growth was suppressed and the weights of the livers were increased by administration of the autoxidized safflower oil. Light microscopic examination revealed no significant changes in the liver, but marked changes in the endoplasmic reticulum and an increase in the number of microbodies in liver cells of the animals after 3 days and accumulation of lipofuscin-like substances in liver cells of animals after 3 months were demonstrated by electron microscopy. Serum glutamic oxaloacetic transaminase and serum glutamic pyruvic transaminase increased in animals of the treated groups. Although triglyceride, cholesterol and phospholipid in the plasma did not show any specific changes, triglyceride increased in the livers of the animals after 3 days but markedly decreased in the animals after 3 months. There was no marked effect on the ^{14}C incorporation into CO_2, but decreased incorporations into cholesterol were demonstrated in the treated animals. The incorporation of ^{14}C into fatty acid was increased by the liver slices of rat after 3 days, but was decreased in the liver slices of animals after 3 weeks and 3 months. These results suggest that the marked decrease in triglyceride in the liver of the 3 month group of animals was partly due to a decreased capacity to synthesize fatty acid in the liver.

INTRODUCTION

For many years, oxidized oil has been known to exhibit deleterious nutritive effects when included in the diet of various mammalian species (1-6). However the effects have ranged from no ill effect or slight suppression of growth to severe toxic effect causing death. Variations of results may be attributed to differences in complexities of materials, the time and amount fed, species differences and other experimental conditions, but details of these problems have not been resolved. Nevertheless the toxic components of oxidized lipids, absorption and transport of these materials and mechanisms of their actions are still important problems.

In order to obtain further information on these problems, we fed autoxidized safflower oil to rats and studied the effect on growth and organ weights. Light and electron microscopic studies were carried out on the organs; blood and liver were collected for chemical analyses.

MATERIALS AND METHODS

The autoxidized safflower oil was prepared by T. Akiya (National Food Research Institute, Yamagata, Japan). The safflower oil was aerated at room temperature under fluorescent lamps for ca. 1 month and had a peroxide value of 465 me/kg. Studies were carried out on four groups of weanling male rats of the Sprague-Dawley strain. One group *N* was fed a normal diet (CA-1, Japan CLEA Co., Tokyo, Japan) ad libitum.

A second group designated *R* was fed daily 10 g of a fat-free diet described by Rice (7). The percentage composition of this diet was: casein, 43.82; sucrose, 13.14; celluflour, 3.29; salt mix (8), 6.57; corn starch, 30.44; vitamin E feed supplement, 1.42 (62.48 IU of vitamin E as *d*-α-tocopherol acetate); vitamin mixture, 1.32. The vitamin mixture supplied 1.25 mg thiamine hydrochloride, 1.25 mg riboflavin, 1.25 mg pyridoxine hydrochloride, 7.0 mg calcium pantothenate, 6.35 mg niacin, 1.60 mg vitamin K, 40.0 μg folic acid, 16.0 μg biotin, 16.0 mg *p*-amino benzoic acid, 16.0 mg *i*-inositol, 160 mg choline chloride and 1.11 g liver powder (1:20) per 100 g diet. In addition to this diet, each rat was fed 1250 U.S.P. units vitamin A and 180 U.S.P. units vitamin D once a week. Analysis of this diet showed that it contained only 0.4% fat.

A third group designated *S* was fed daily 10 g of the Rice diet and administered 1 ml

[1]Presented at the JOCS-AOCS Joint Meeting, Los Angeles, April 1972.

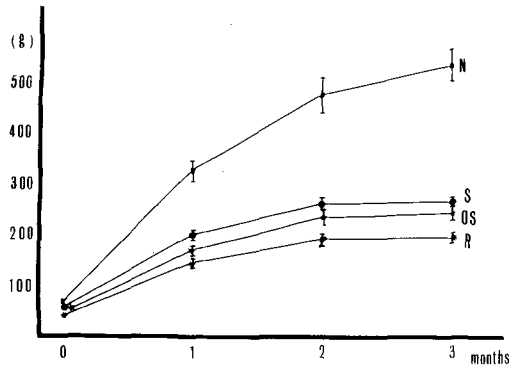

FIG. 1. Body weight. Each point represents mean ± SD for 15 animals.

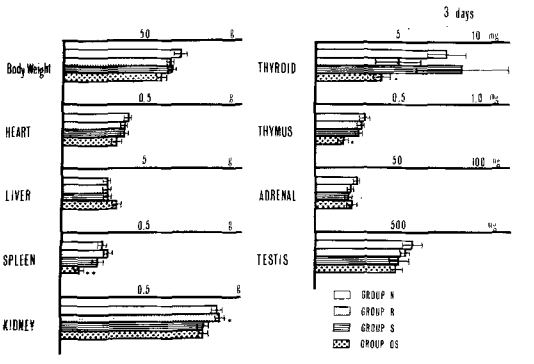

FIG. 2. Body and organ weight. M ± SE for 15 animals. * = $P < 0.05$; ** = $P < 0.01$. Compared to group S.

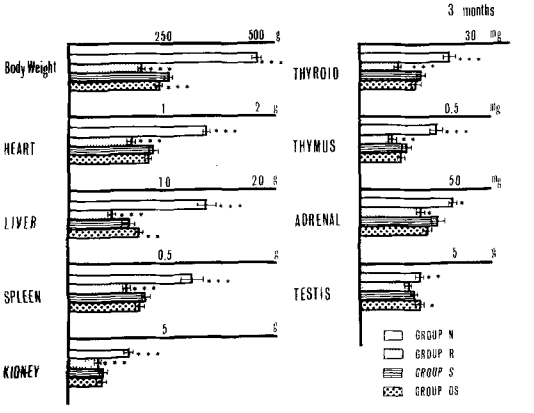

FIG. 3. Body and organ weight. M ± SE for 15 animals. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. Compared to group S.

safflower oil by intubation; a fourth group designated *OS* was fed daily 10 g of the Rice diet and administered 1 ml autoxidized safflower oil by intubation.

The animals were housed in individual cages, and at intervals of 3 days, 3 weeks and 3 months, 15 animals of each group were killed by drawing blood from the aorta under ether anesthesia; their organs were removed and immediately weighed. Sections of the organs were fixed for histological examination by light and electron microscopy. For electron microscopic examination, samples of livers were fixed for 2 hr at 4 C in 1.19% glutaraldehyde buffered with 0.1 M phosphate buffer at pH 7.4. They were held overnight in 0.1 M phosphate buffer at pH 7.4 plus 0.14 M sucrose. Postosmification was done in 1% osmium tetroxide for 2 hr at 4 C. Graded alcohol was used for dehydration and Epon 812 for embedding. Thin sections were stained with lead citrate and uranium acetate. Selected areas were photographed in 100-U type electron microscope (JEOL).

The total lipid was extracted by the procedure of Folch et al. (9) and examined for cholesterol (10), triglyceride (11) and lipid phosphorous (12). Blood glucose was determined by the autoanalyzer procedure adapted from the method of Hoffman (13). Serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) were determined by a modified method of Reitman and Frankel (14). Alkaline phosphatase was determined by the method of Bossey et al. (15).

The incorporation of ^{14}C-acetate into CO_2, cholesterol and total fatty acid by liver slices was determined by a modified method of Rodbell (16). Five hundred milligrams of liver slices were prepared from the animals in the groups treated as described and were incubated in a flask which contained 2.5 ml bicarbonate buffer pH 7.4 and ca. 150,000 cpm 1-^{14}C-sodium acetate (specific activity 49.3 mc/mM, Radiochemical Center, Amersham, England). For CO_2 counting, the flask had a glass well that contained a cylinder of Whatman No. 1 paper rolled from 2 x 8 cm strip. After gassing with 95% O_2/5% CO_2, the flasks were capped with rubber stoppers. Incubations were carried out for 2 hr at 37 C in the metabolic shaker, followed by the incubation in cold water (0-4 C) for 15 min. At the end of the incubation, 0.2 ml Hyamine-10X (Packard Instrument Co., Downers Grove, Ill.) was injected into the filter paper and 0.25 ml 1N sulfuric acid into the incubation medium, followed by shaking for 30 min. The CO_2 was counted in a scintillation solution consisting of PPO-POPOP in toluene by the procedure of Rodbell (16). For the determination of ^{14}C incorporation into cholesterol and fatty acid, the liver slices and incubation medium were transferred to the tube and saponified by 1 ml 50% KOH under 70-80 C for 1.5 hr. After 3.75 ml water was

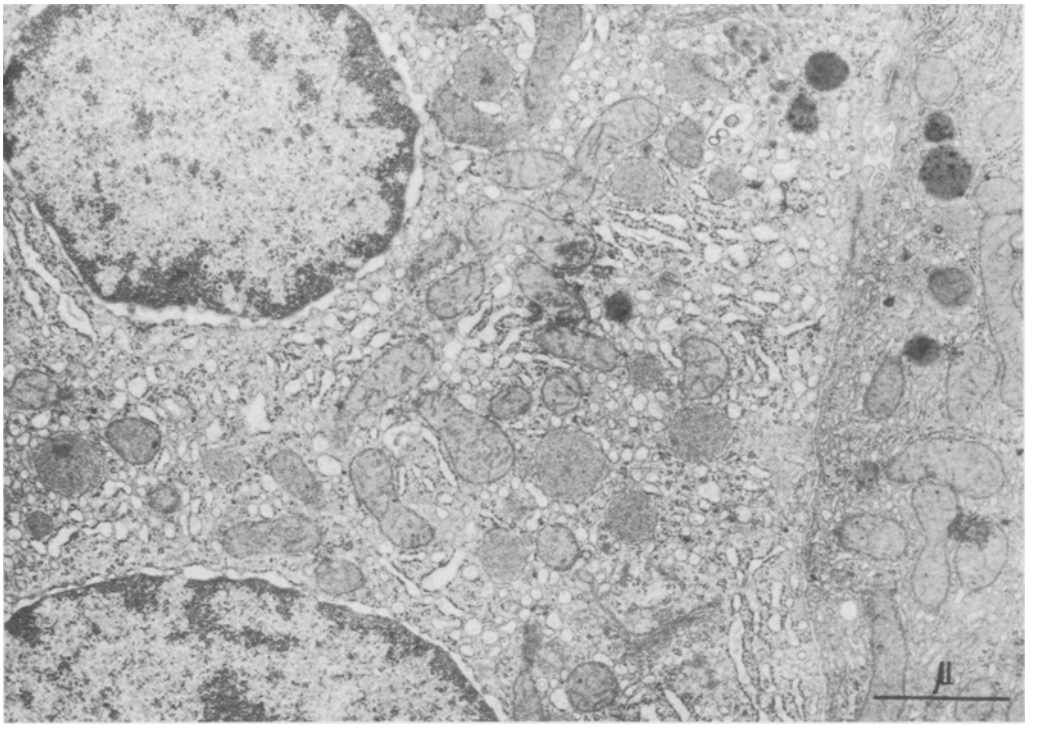

FIG. 4. Electron micrograph of parts of hepatocytes from a rat of the OS group treated for 3 days. Major changes were involved in rough endoplasmic reticulum and in increased size and number of microbodies.

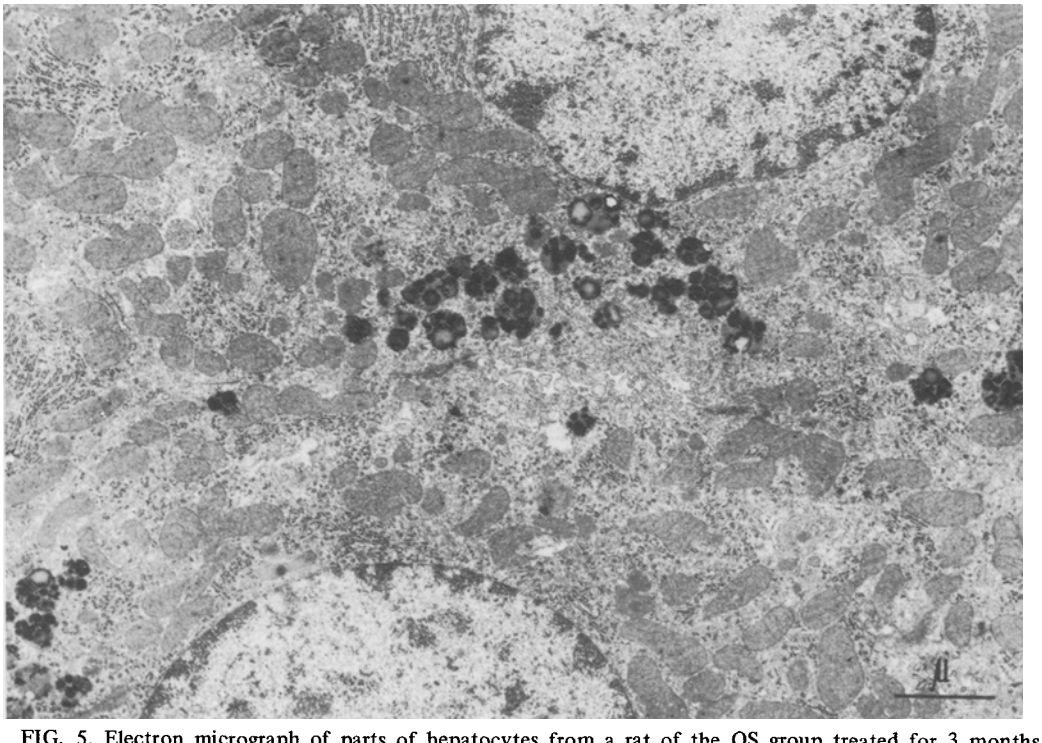

FIG. 5. Electron micrograph of parts of hepatocytes from a rat of the OS group treated for 3 months. Numbers of lysosomes and high density granular bodies that were lipofuscin-like substances were markedly increased around bile canaliculi.

TABLE I

Effects of Autoxidized Safflower Oil on SGOT, SGPT and Alkaline Phosphatase[a]

Enzyme	Normal (N group)	Rice diet (R group)	Safflower oil (S group)	Autoxidized safflower oil (OS group)
3 weeks				
SGOT (KU)[b]	61.0 ± 5.3[c]		52.8 ± 4.1	59.4 ± 4.1
SGPT (KU)	13.3 ± 1.6		12.5 ± 1.0	28.2 ± 2.5***
Alkaline phosphatase (BLU)	18.0 ± 2.1		17.5 ± 2.8	21.7 ± 2.7
3 months				
SGOT (KU)	73.2 ± 3.8**	136.7 ± 11.2**	89.3 ± 4.1	182.8 ± 7.3***
SGPT (KU)	30.1 ± 2.6	48.5 ± 44.1**	26.9 ± 2.3	84.0 ± 6.4***
Alkaline phosphatase (BLU)	4.2 ± 0.3	5.4 ± 0.4	4.9 ± 0.9	4.0 ± 0.4

[a]SGOT = serum glutamic oxaloacetic transaminase; SGPT = serum glutamic pyruvic transaminase. * = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$. Compared to group S.

[b]KU = Karumen Unit.

[c]Mean ± SE for five animals.

[d]BLU = Bessey-Lowery Unit.

added, the sample was acidified to pH 3.0 with sulfuric acid. Cholesterol and fatty acid were extracted with petroleum ether and ethyl ether and separated by thin layer chromatography (petroleum ether-ethyl ether-acetic acid 50:50:1). The bands of fractions were scraped directly from plates into vials of scintillation solution consisting of PPO-POPOP in dioxane-water solution for counting (17).

RESULTS AND DISCUSSION

The growth curves of each group are shown in Figure 1. The N group, which was fed a normal diet ad libitum, gave a normal growth curve. Among the three groups fed restricted amounts of the diets, the S group had the best growth, the OS group was next and the R group was the poorest. Because the S group was supplied calories and some essential fatty acids, they showed better growth than the R group. The diet of the OS group also contained calories and essential fatty acids, but the autoxidized components apparently suppressed growth. Among these four groups, statistical significant differences were demonstrated.

By the fourth day, the R and S groups did not show any marked physiological change other than body weight. However the OS group

TABLE II

Effects of Autoxidized Safflower Oil on Amounts of Triglyceride, Cholesterol and Phospholipid in Liver[a]

Lipid class	Normal (N group)	Rice diet (R group)	Safflower (S group)	Autoxidized safflower oil (OS group)
3 days, mg/100 g				
Cholesterol	343.3 ± 14.6[b]	367.5 ± 36.5	316.4 ± 15.0	265.0 ± 21.1
Triglyceride	121.6 ± 12.4	132.0 ± 13.6	118.6 ± 18.8	149.0 ± 11.5
Phospholipid-P	91.5 ± 5.6	79.5 ± 6.2	80.8 ± 2.1	81.5 ± 4.1
3 weeks, mg/100 g				
Cholesterol	255.4 ± 13.6		256.0 ± 11.6	235.2 ± 15.7
Triglyceride	1024.0 ± 222.2		762.0 ± 83.0	596.0 ± 73.0
Phospholipid-P	88.8 ± 4.6*		102.4 ± 3.0	104.8 ± 4.6
3 months, mg/100 g				
Cholesterol	581.6 ± 81.8	351.5 ± 16.3*	398.6 ± 11.9	304.0 ± 14.3***
Triglyceride	2016.6 ± 370.8	1027.8 ± 384.4	1568.2 ± 350.0	254.8 ± 21.2**
Phospholipid-P	100.2 ± 3.6	104.3 ± 1.9	110.8 ± 6.0	109.4 ± 7.3

[a]* = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$. Compared to group S.

[b]Mean ± SE for five animals.

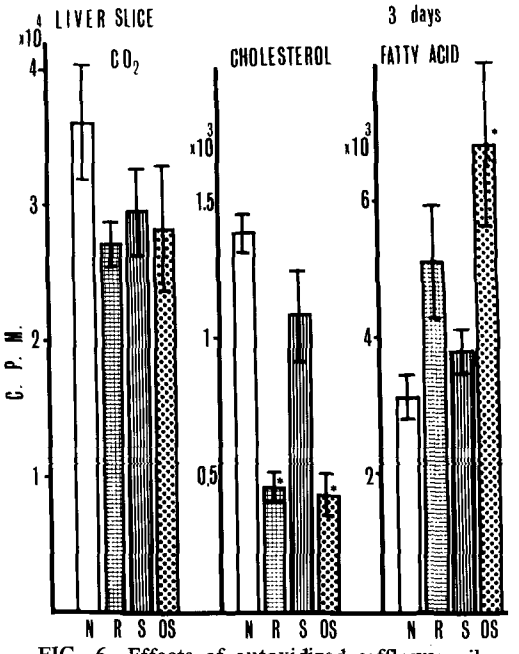

FIG. 6. Effects of autoxidized safflower oil on incorporation of 1-^{14}C-acetate into CO_2, cholesterol and fatty acid. M ± SE for five animals. * = $P < 0.05$. Compared to group S.

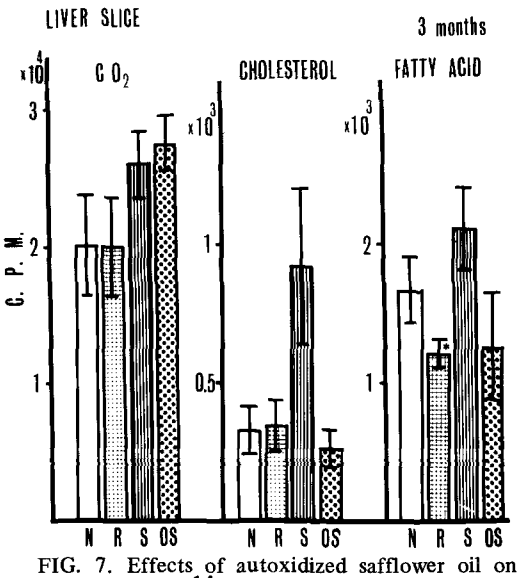

FIG. 7. Effects of autoxidized safflower oil on incorporation of 1-^{14}C-acetate into CO_2, cholesterol and fatty acid. M ± SE for five animals. * = $P < 0.05$. Compared to group S.

revealed such toxic effects as decreased body weight, rough and coarse hair, diarrhea, and dirtiness and moisture around the mouth and anal areas. At autopsy major changes were demonstrated in the gastrointestinal tract, such as erosion of the stomach, dilatation of the duodenum, jejunum and colon. These symptoms gradually disappeared as the feeding was continued.

Figure 2 shows the body and organ weights of the groups after 3 days. In the OS group, with decreased body weight, weights of spleen, thyroid and thymus decreased but weight of liver increased. These observations are generally in accord with the similar studies by Kaunitz et al. (5).

Figure 3 shows the results of the treatment for 3 months on the body and organ weights. Although the body weight was decreased, liver weight increased in the OS group

Histological Observation

Of the groups treated for 3 days, the light microscopic examination of the spleen showed a marked change only in the OS group. In the spleen of the weanling rats extramedullary hematopoesis, erythoblastens and immature granular cells were observed. However these were not apparent in the OS group. The decreased weight of thymus in the OS group was due to cortical atrophy, which was histologically demonstrated.

The light microscopic examination of the liver of the OS group did not show any specific change, but the electron microscopic examination revealed marked changes in the liver cells of the OS group (Fig. 4). The major changes were involved in the rough endoplasmic reticulum with disappearance of arrangement in parallel array, fragmentation, dilatation, prominent detachment of ribosomes and transformation to smooth endoplasmic reticulum. The size and number of microbodies increased in the OS group. Similar changes of microbodies were reported to be induced by the administration of such low cholesterolemic agents as ethyl chlorophenoxybutyrate (18,19) (CPIB) and biphenylyl methylvaleric acid (20). These results suggested a relationship between lipid metabolism and lipid peroxide administration.

In the groups treated for 3 months, the histological examination of liver of the OS group revealed no fatty metamorphosis of hepatocytes and no other marked changes. As electron microscopic examinations of the 3 months group were undertaken by the material previously fixed for routine light microscopic examination, the detailed structural analysis was not available; but in the OS group the number of lysosomes and high density granular bodies, which were lipofuscin-like substances (21), were observed to be markedly increased around the bile canaliculi (Fig. 5). Lipofuscins (22) are considered to be derived from endogenous lipids by oxidation. However the result

strongly suggested that lipofuscin-like substances could also originate, directly or indirectly, from the autoxidized safflower oil, which was exogenously administered for 3 months.

Blood Chemical Analysis

Although serum glucose, cholesterol and triglyceride showed no specific change during the 3 months, administration of the autoxidized safflower oil caused a marked effect on the SGPT and SGOT (Table I).

After 3 weeks, SGPT in the OS group was elevated, but SGOT and alkaline phosphatase showed no differences between the S group and the OS group. After 3 months, the R group showed a higher SGOT and administration of safflower oil lowered the SGOT level, indicating that the elevation of SGOT might be due to the essential fatty acid deficiency. However the OS group showed the highest SGOT level. This result suggested that, when autoxidized oil was contained in the safflower oil, safflower oil could not lower the SGOT level and this autosidized oil was the effective substance that increased the SGOT level. Similar results were obtained in the SGPT level.

Although appearances of the rats of the OS group treated for 3 months were not severe, the enlargement of liver and elevation of SGPT and SGOT level strongly suggested liver disfunction.

Lipid Analysis of Liver

Table II shows the amount of cholesterol, triglyceride and phospholipid in the liver of the groups treated for 3 days, 3 weeks and 3 months. Though some differences were shown in the amount of cholesterol and triglyceride in the liver between S and OS groups treated for 3 days and 3 weeks, the results of 3 months showed a marked decrease in the amount of triglyceride. These results indicated that the enlarged and heavy livers of the OS group treated for 3 months were not due to an accumulation of excessive fat.

Incorporation of 1-^{14}C-Acetate into CO_2, Cholesterol and Fatty Acid by Liver Slice

The result of 3 days of treatment (Fig. 6) showed no difference in the CO_2 production. The incorporation into cholesterol decreased and the incorporation into fatty acid increased in the R group and the OS group.

The administration of autoxidized oil for 3 months resulted in the changes in the incorporation of ^{14}C into cholesterol and fatty acid (Fig. 7). The S group was the highest and the R and OS groups showed the lowest incorporation. If safflower oil administration were effective on the increased incorporation of ^{14}C, the autoxidized safflower oil might render this effect. The amount of triglyceride in the liver and the ^{14}C incorporation into fatty acid were decreased from 3 days to 3 months, demonstrating a correlation between these in vitro results of the incorporation into fatty acid and the amount of triglyceride in the liver.

Several investigators report (23-25) that lipid peroxide inhibits many enzyme activities, and the mechanisms of the changes in the amount of triglyceride in the liver might be partly due to the decreased capacity of this organ for fatty acid synthesis.

The early effect of autoxidized safflower oil administration consisted of changes in the endoplasmic reticulum and the increased incorporation of ^{14}C into fatty acid. The R group also showed the increased incorporation of ^{14}C into fatty acid but no pronounced changes in the endoplasmic reticulum, indicating that the mechanism of increased incorporation of the OS group was different from that of the R group. The changes of endoplasmic reticulum in the OS group treated for 3 days were similar to the findings demonstrated by the treatment of carbon tetrachloride (26) and phenobarbital (27,28). These chemicals are also peroxidation agents or stimulants of drug metabolizing enzymes. The microsomes of the liver are the site of oxidative metabolism of many compounds, steroids and drugs (29). Bernhard et al. noted an increased synthesis of hepatic arachidonate in tocopherol-deficient rats, which they attributed to a lack of control of the oxidative processes of fatty acid chain elongation and desaturation in the deficient of antioxidant (30). Witting (31,32) demonstrates not only an increase in arachidonate in the tocopherol-deficient rat fed high levels of both linoleate and linolenate, but also increased synthesis of arachidonate and of pentanoic acid using intraperitoneally injected 1-^{14}C acetate. The early stimulant effect of autoxidized lipid for fatty acid synthesis could be in accord with these observations; however long term administration of autoxidized safflower oil caused the accumulation of a large amount of peroxidic material inhibiting the enzyme activity. In order to elucidate these mechanisms more clearly, further and more detailed research will be needed.

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REFERENCES

1. Matsuo, N., J. Biochem. (Japan) 41:647 (1954).

2. Kaneda, T., H. Sakurai and S. Ishii, Bull. Jap. Soc. Sci. Fish. 20:658 (1954).
3. Kaunitz, H., C.A. Slanetz and R.E. Johnson, J. Nutr. 55:577 (1955).
4. Andrews, J.S., J.F. Mead and W.H. Griffith, Fed. Proc. 15:918 (1956).
5. Kauntiz, H., C.A. Slanetz, R.E. Johnson, H.B. Knight, D.H. Saunders and D. Swern, JAOCS 33:630 (1956).
6. Privett, O.S., and R. Cortesi, Lipids 7:780 (1972).
7. Rice, E.E., W.D. Warner, P.E. Mone and C.E. Poling, J. Nutr. 61:253 (1957).
8. Jones, J.H., and C. Foster, Ibid. 24:245 (1942).
9. Folch, J., M. Lees and F.H. Sloane-Stanley, J. Biol. Chem. 226:497 (1957).
10. Zak, B., Amer. J. Clin. Path. 27:583 (1957).
11. Van Handel, E., Clin. Chem. 7:249 (1961).
12. Marrinetti, G.V., J. Lipid Res. 3:1 (1962).
13. Hoffman, W.S., J. Biol. Chem. 120:51 (1937).
14. Reitman, S., and S. Frankel, Amer. J. Clin. Path. 28:56 (1957).
15. Bessey, O.A., O.H. Lowry and M.J. Brock, J. Biol. Chem. 164:321 (1964).
16. Rodbell, M., Ibid. 239:375 (1964).
17. Snyder, F., Anal. Biochem. 9:183 (1964).
18. Svoboda, D., H. Grady and D.L. Azarnoff, J. Cell Biol. 35:127 (1967).
19. Legg, P.G., and R.L. Wood, Ibid. 45:118 (1970).
20. De La Iglesia, F.A., J.C. Sosa-Lucero and G. Lumb, "Proceedings of the European Society for the Study of Drug Toxicity," Vol. 11, Exerpta Media, Amsterdam, 1970, p. 191.
21. Fawcett, D.W., "The Cell," Saunders Co., Philadelphia, 1967, p. 282.
22. Walter, J.B., and M.S. Isrel, "General Pathology," J.&A. Churchill Ltd., London, 1963, p. 870.
23. Tappel, A.L., Fed Proc. 24:73 (1965).
24. Little, C., and P.J. O'Brien, Biochem. J. 106:419 (1968).
25. Bernheim, F., K.M. Wilbur and C.B. Kenaston, Arch. Biochem. Biophys. 38:177 (1952).
26. Rouiller, C.H., "The Liver," Vol. II, Academic Press, New York, 1964, p. 335.
27. Stäubli, W., R. Hess and E.R. Weibel, J. Cell Biol. 42:92 (1969).
28. Smucker, E.A., and M. Arcasoy "International Review of Experimental Pathology," Vol. 7, Academic Press, New York, 1969, p. 305.
29. Williams, R.T., "Detoxication Mechanism," Chapman and Hall, London, 1959, p. 717.
30. Bernhard, K., S. Leisinger and W. Pedersen, Helv. Chim. Acta 46:1767 (1963).
31. Witting, L.A., Lipids 2:109 (1967).
32. Witting, L.A., in "Progress in the Chemistry of Fats and Other Lipids," Vol. 9, Edited by R.T. Holman, Pergamon Press, New York, 1971, p. 536.

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Antagonistic Effects of *Myxococcus xanthus* on Fungi: II. Isolation and Characterization of Inhibitory Lipid Factors

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ABSTRACT

The chemical composition of the lipophilic excretion of *Myxococcus xanthus* inhibitory to the germination of fungal spores and growth has been investigated. The inhibitory effect was attributed to a mixture of fungistatically acting fatty acids and a component of antibiotic character. The fatty acid mixture has been fully characterized and found to constitute a mixture of saturated (68%) and unsaturated (32%) structures in the C_{13}-C_{17} range. The major part is methyl-branched of the *iso*-type, with 13-methyltetradecanoic acid being the main component (33% of the total). The fungistatic activity of the fatty acid mixture on spore germination is attributed to the structures with *iso*-configuration. The presence of unsaturation is of minor importance. Observed morphological changes of the spores and hyphae in the presence of *iso*-fatty acids suggest that they act on the plasma membrane.

INTRODUCTION

Myxobacteria are terrestrial organisms which are ubiquitous inhabitants of normal soil, bark, and decaying plant material (1). As described in a previous paper (2), *Myxococcus xanthus* secretes into the culture medium organic substances inhibitory to the germination of spores and the growth of fungi. The isolation and characterization of these excreted factors are important, as they possibly may be involved in the phenomenon known as soil mycostatis.

In an early stage of the investigation of the chemical background of these findings, it was found that the responsible components readily dissolved in organic solvents such as ethyl acetate, chloroform or benzene. Recently, Schröder and Reichenbach (3) reported an analysis of the fatty acids of vegetative cells and myxospores of *Stigmatella aurantiaca*. A saturated branched C_{15} acid (~35%) and a monounsaturated C_{16} acid (~20%) were identified as the main fatty acids. No effort was made to locate the position of the branch (assumed to be a methyl group) or the double bond.

This paper deals with extracellular material in the culture medium and not with extracts of cells.

EXPERIMENTAL PROCEDURES

The cultivation of *M. xanthus* has been described previously (2).

Germination experiments: Two wetted and sterilized strips (10 x 10 mm) of cellophane (PT 300) were placed on a sterile glass filter (Jena G1) in a small petri dish half filled with 3 ml test solution. The nutrient medium consisted of glucose (2.0 g) KNO_3 (2.0 g), KH_2PO_4 (2.5 g), $MgSO_4 \cdot 7H_2O$ (1.26 g) and Tween 80 (0.1 ml) in distilled water (1000 ml). The preparations to be examined were added to the medium as methanol solutions. All media had a methanol concentration of 1.0% and pH 6.5. The strips were inoculated with conidia of *Fusarium roseum* (40,000 conidia per strip) from cultures grown for 10 days at 25 C. The strips were removed after 3 and 4 hr incubation at 25 C, mounted on slides, and treated with lactophenol to stain and kill the conidia. The proportions of germinated and nongerminated conidia were then determined in the microscope. On each strip at least 400 randomly chosen conidia were examined.

Analytical gas chromatography (GC): A 50 m long stainless steel capillary tube, ID 0.01 in. (Golay capillary column type R) coated with polypropylene glycol was used. A Perkin-Elmer model 900 gas chromatograph equipped with a standard capillary injector block, split no. 3, and flame ionization detectors was employed. Helium was used as carrier gas, and the gas flow was ca. 3 ml/min at room temperature.

Preparative GC: The column used had a length of 8 m and an internal diameter of 8 mm. It was filled with Versamid 900 (6%) on Gaschrom Z (80-100 mesh) and was initially kept at 275 C during 24 hr in a stream of nitrogen. The chromatography was performed at 190 C on a modified Aerograph (model A-700) gas chromatograph operated manually under isothermal conditions. The flow of helium carrier gas was 100 ml/min, and the vapors were condensed in standard Autoprep flasks filled with stainless steel turnings. The turnings were moistened with 0.5 ml chloroform and the flasks immersed in a cold bath at -80 C.

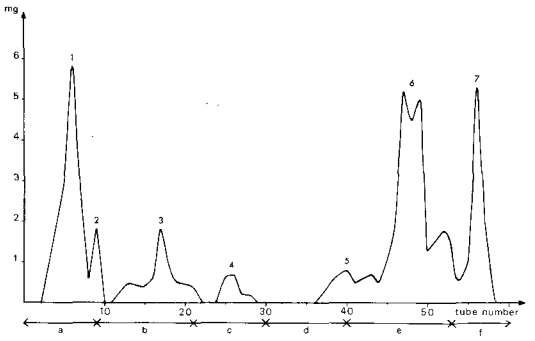


FIG. 1. Liquid chromatogram of the lipophilic excretion of *M. xanthus* on silicic acid. Tube contents are shown as a function of tube number. *a* benzene-ethyl acetate (3:1 v/v), *b* benzene-ethyl acetate (1:1 v/v), *c* ethyl acetate, *d* ethyl acetate-acetone (1:1 v/v), *e* acetone, *f* methanol. Three liters of culture medium.

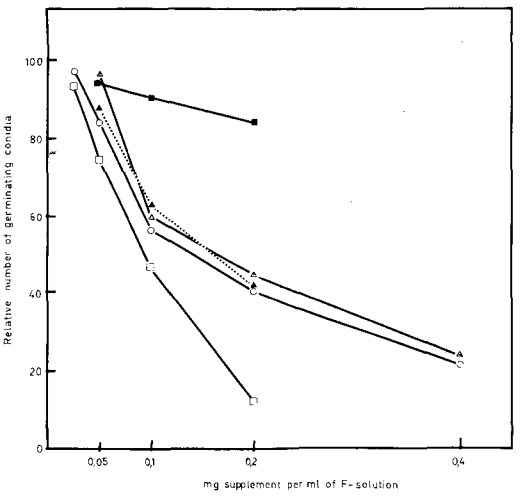


FIG. 2. Relative number of germinating conidia of *F. roseum* after 3 hr incubation at 25 C. Control = 100. Supplement to F-solution: □ lipophilic extract of cell free culture solution of *M. xanthus*, ○ natural fatty acid mixture, △ saturated moiety of natural fatty acid mixture, ▲ unsaturated moiety of natural fatty acid mixture, ■ neutral fraction of the lipophilic extract.

Mass spectrometry (MS): The GC-MS combination instrument described by Stallberg-Stenhagen et al. (4) was used. The temperature of the ion source was 200 C, and the electron energy 70 eV.

Catalytic hydrogenation: In a typical experiment, 4.5 mg of the unsaturated methyl ester mixture was dissolved in 2 ml *n*-heptane in a 25 ml flask. One milligram of Adam's catalyst ($PtO_2 \cdot H_2O$) was added and the flask filled with hydrogen, stoppered, and shaken for 15 min at 25 C. The suspension was centrifuged prior to analysis.

Oxidative degradation: The procedure was illustrated by the oxidative degradation of component XIV; 0.5 mg XIV was dissolved in 50 µl glacial acetic acid in a small test tube. One and eight-tenths milligrams finely pulverized potassium permanganate was added and the mixture heated for 15 min at 40 C. One-tenth milliliter of a solution of sulphur dioxide in water (10%) was added, followed by one drop of diluted sulphuric acid (5%). The mixture was heated on the waterbath until colorless. One-tenth milliliter dichloromethane was added; after shaking and subsequent centrifugation the aqueous layer was discarded and the organic phase washed with water. After evaporation the residue was esterified with diazomethane in ether. The reaction product was investigated on the combined GC-MS.

Preparation of branched chain reference material. Synthesis of 12-methyltridecanoic acid: This previously known acid, see e.g. (5), was prepared via a mixed anodic coupling (Kolbe electrosynthesis) of 4-methylpentanoic acid (Fluka AG, Bucks, Switzerland) and methyl nydrogen decan-1,10-dioate (6). There was obtained 13.5 g of the desired methyl ester of bp 136-140 C, 1 mm, from 64.0 g half ester and 34.4 g 4-methylpentanoic acid. The methyl ester was hydrolyzed and the free acid recrystallized from light petroleum (bp 60-85 C). Yield 10.7 g, mp 52.2-52.8 C of free acid.

13-Methyltetradecanoic acid: This previously known acid (7) was prepared from 12-methyltridecanoic acid by chain lengthening (Arndt-Eistert reaction). The diazoketone obtained from 5.0 g of 12-methyltridecanoic acid was rearranged in methanol in the presence of commercial silver oxide. The crude methyl 13-methyltetradecanoate (5.1 g) was chromatographed on 50 g silicic acid (Mallinckrodt, 100 mesh) with ether-light petroleum (bp 40-60 C) (1:50 v/v). Free acid (2.1 g) mp 49.7-50.3 C was obtained after hydrolysis and crystallization from light petroleum (bp 40-60 C).

RESULTS

Fractionation of the crude inhibitory material: After 4 days of incubation at 30 C, the cells (5.2 g dry weight) were removed from 3000 ml of culture solution by centrifugation. The solution was shaken immediately with five 200 ml portions of ethyl acetate (Analar grade), and the combined organic extracts evaporated to dryness at reduced pressure (25 C). One hundred thirty-five milligrams of brownish viscous residue with a strong odor characteristic of Myxobacteria was obtained. The extract was triturated with 25 ml benzene for 1 hr and the suspension centrifuged. The benzene soluble material weighed 94 mg (69.7%). This was

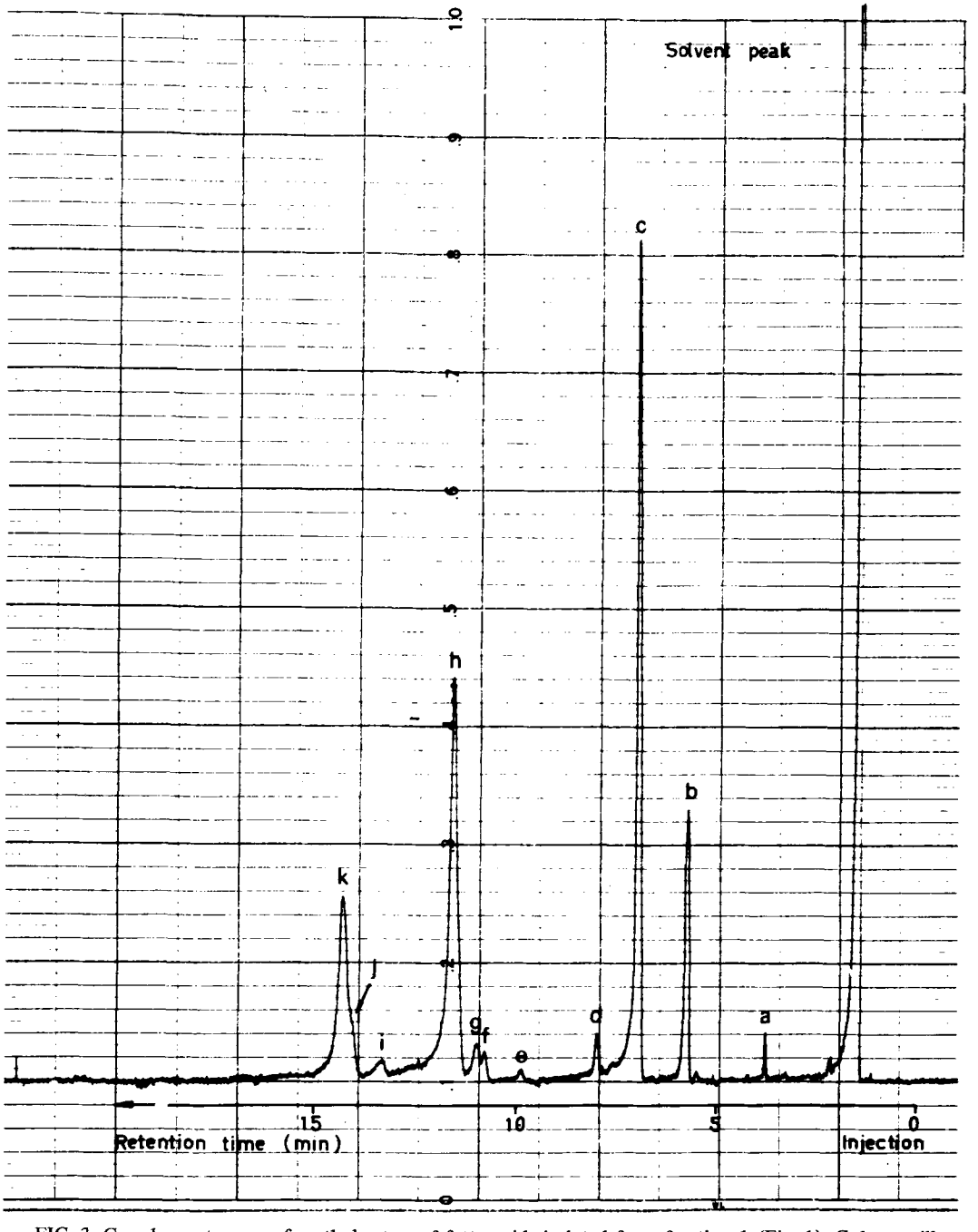
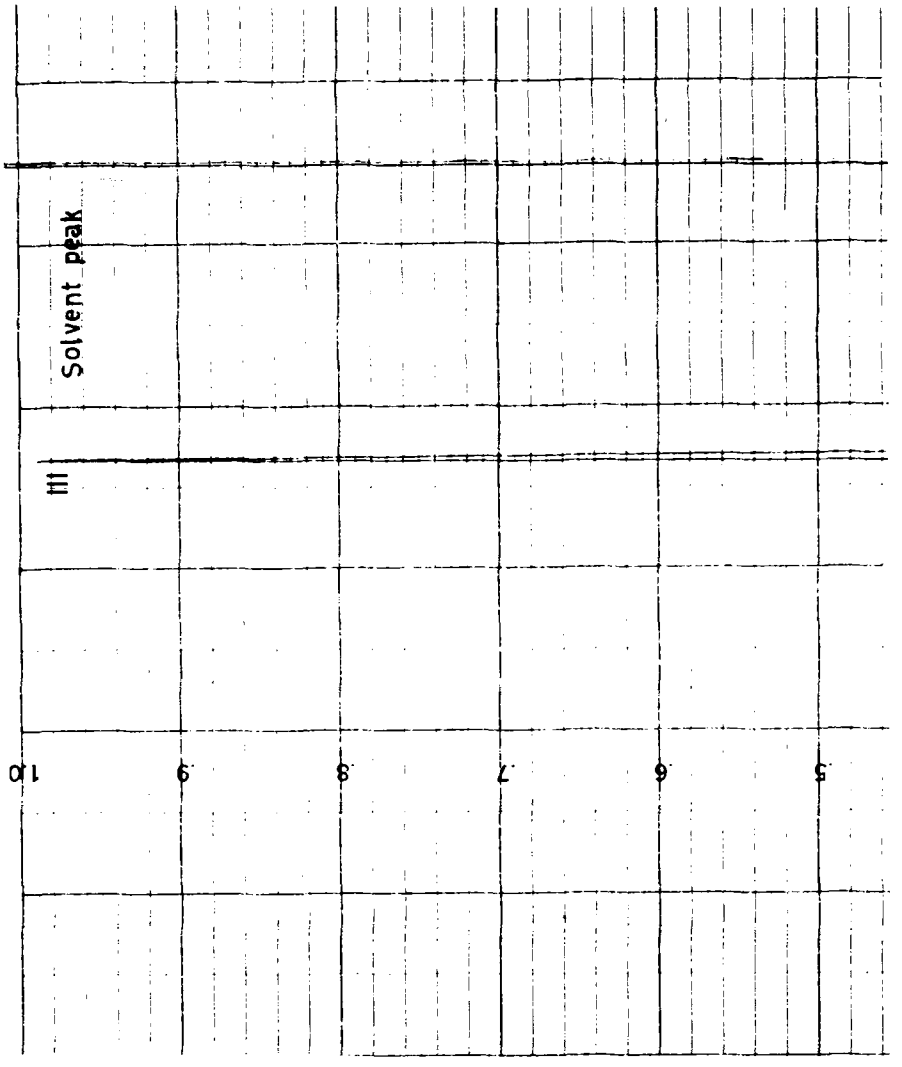


FIG. 3. Gas chromatogram of methyl esters of fatty acids isolated from fraction 1 (Fig. 1). Golay capillary column type R with polypropylene glycol as stationary phase at 185 C with *n*-heptane as solvent.

chromatographed on 5 g silicic acid activated at 110 C overnight (Mallinckrodt 200-325 mesh).

The polarity of the eluant was increased stepwise as follows—20 ml benzene-ethyl acetate (3:1 v/v), 60 ml benzene-ethyl acetate (1:1 v/v), 45 ml ethyl acetate, 50 ml light petroleum (bp 40-60 C)-acetone (1:1 v/v), 65 ml acetone, and 50 ml methanol. Five milliliter fractions were collected in preweighed flasks. The solvents were removed in a stream of nitrogen; 91% of the material was eluted (Fig. 1). The fractions were combined as indicated (1-3) and


Solvent peak
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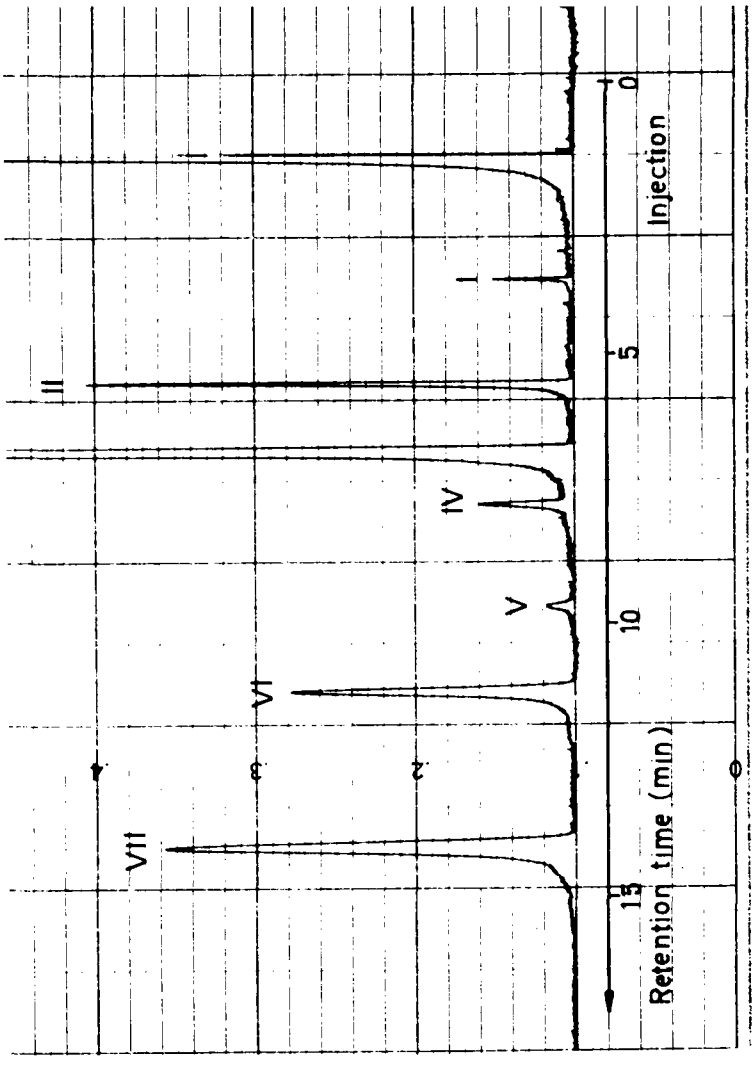

FIG. 4. Gas chromatogram of methyl esters of saturated fatty acids isolated from fraction 1. Conditions identical with those in Figure 3.

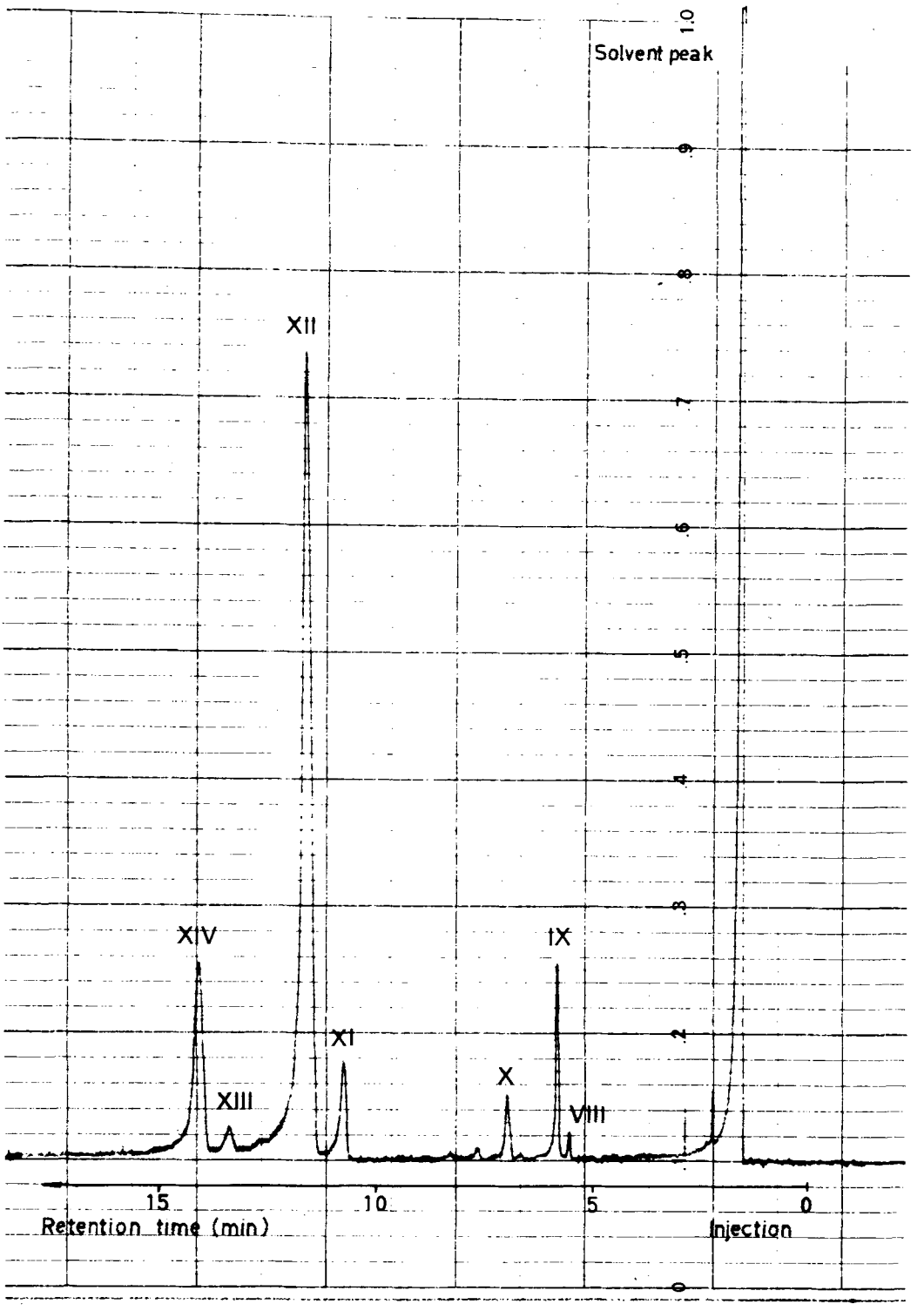

FIG. 5. Gas chromatogram of methyl esters of unsaturated fatty acids isolated from fraction 1. Conditions identical with those in Figure 3.

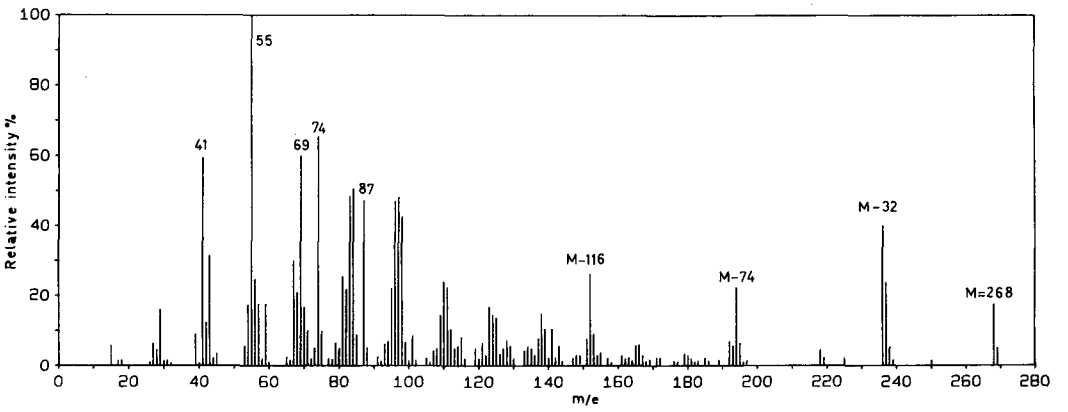

FIG. 6. Mass spectrum of component XII.

tested for inhibitory activity on the germination of *Fusarium* conidia. Fractions 1 and 3 exhibited such activity; whereas, the rest of the material had practically no effect on germination. This paper deals with lipid fraction 1; whereas, fraction 3 will be dealt with in a later paper.

An IR spectrum of fraction 1 indicated the presence of free carboxylic acid groups by characteristic absorptions at 2700-2500 cm^{-1} and 1700 cm^{-1}. The material (86 mg) was dissolved in a small volume of methanol and titrated with sodium methoxide in methanol to pH 9 and subsequently distributed between water and ether. Acidification of the aqueous phase, followed by repeated extraction with ether, afforded the acidic material (64 mg, 77%) in a state sufficiently pure for further analysis. The tests for activity on fungal development indicated that the acidic components accounted for a strong inhibition, while the neutral fraction had practically no such effect (Fig. 2). An IR spectrum of the purified acids showed, in addition to the characteristic absorptions previously mentioned, weak absorption at 1650 cm^{-1} (-C=C-stretching vibrations) given by, for example, unsaturated fatty acids of the nonconjugated type (8).

Fractionation and identification of the fatty acids: The acids subsequently were esterified by means of diazomethane and subjected to analysis on a capillary GC column (Fig. 3). At least 11 components, designated *a-k*, are distinguishable.

The fatty acid methyl esters were separated into saturated and unsaturated fractions by adsorption chromatography on silicic acid impregnated with silver nitrate (9,10). In a typical experiment 177 mg methyl esters were chromatographed on 5.0 g argentated silicic acid. About two-thirds of the material (119 mg) was saturated and eluted with light petroleum (bp 60-70 C)-ether (50:1 v/v) (I-VII, Fig. 4) while the rest (55 mg), eluted with light petroleum-ether (5:1 v/v) consisted mainly of monounsaturated methyl esters (VIII-XIV, Fig. 5).

It is evident from Figures 3-5 that peak *b* in Figure 3 is composed of three structures (II, VIII and IX), peak *c* of III and a small amount of X, and peak *h* of the two structures VI and XII. By adding normal chain C_{13}, C_{14}, C_{15}, and C_{16} methyl esters (Fluka AG, Switzerland) to a sample of the natural saturated methyl esters and by rechromatographing this mixture, peaks II, IV and VI (Fig. 4) were recognized as due to methyl tetradecanoate, methyl pentadecanoate and methyl hexadecanoate, respectively. Mass spectra of the molecules confirmed the findings.

The mass spectra of I, III, V and VII were found to be very similar to those of the methyl esters of normal C_{13}, C_{15}, C_{16} and C_{17} acids. However, as already shown (Fig. 4), the GC retention times were considerably shorter. This suggested that the 4 components belong to the series of *iso*-fatty acids, i.e., acids possessing a terminal isopropyl group (11). The mass spectra and retention times of synthetic (see Experimental Procedures) methyl esters of *iso*-C_{13}, C_{15}, C_{16} and C_{17} acids proved identical with those of components I, III, V and VII, respectively. The findings constituted proof as to the *iso*-configuration.

A sample of the unsaturated material (Fig. 5) was subjected to total hydrogenation. The GC now showed only 4 peaks which were identified by their retention times as the methyl esters of tetradecanoic-, *iso*-pentadecanoic-, hexadecanoic- and *iso*-heptadecanoic acid. The unsaturated acids thus possessed carbon skeletons of the same type as the saturated fatty acids.

Components IX, X, XI, XII and XIV (Fig. 5) were isolated by means of preparative GC. The

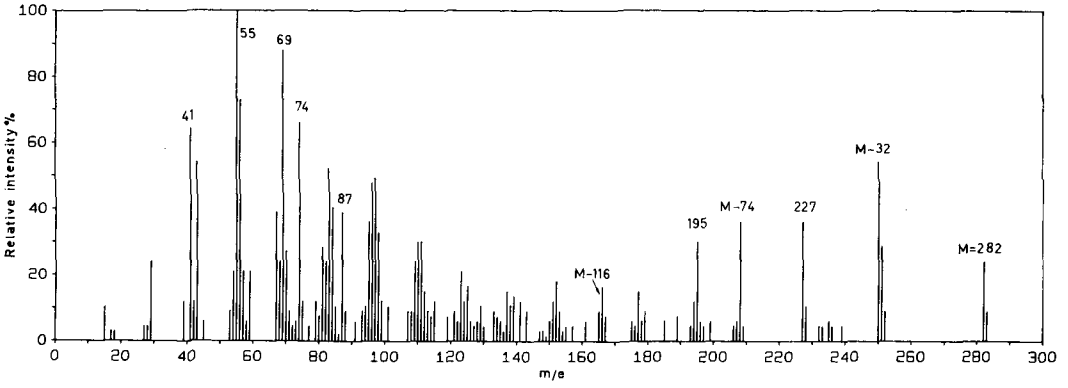

FIG. 7. Mass spectrum of component XIV.

mass spectrum of XII, the main unsaturated component, was reproduced in Figure 6. It indicated a molecular weight of 268 which was that of the methyl ester of a monounsaturated hexadecanoic acid. Characteristic peaks of even mass were observed at m/e 236 (=M-32), m/e 194 (=M-74), and m/e 152 (=M-116). Such peaks also were present in the mass spectrum of, for example, methyl oleate and may be regarded as characteristic for monounsaturated long chain methyl esters (11).

After catalytic hydrogenation as discussed above, sample XII showed a retention time identical with that of methyl hexadecanoate (VI, Fig. 5). The double bond of XII was located by analysis of the products obtained on oxidative degradation with the aid of potassium permanganate. Identification of the fragments obtained with the GC-MS combination revealed the presence of methyl *n*-pentanoate and dimethyl undecan-1,11-dioate. Hence, XII had the double bond in 11:12 position. The absence of absorption at 965 cm^{-1} in the IR (out-of-plane = CH deformation vibrations) generally was taken as proof of *cis*-configuration in esters of long chain unsaturated fatty acids (10). Since XII did not absorb at 965 cm^{-1}, the structure *cis*-hexadec-11-enoic acid is assigned to component XII.

The mass spectrum of XI indicated a mol wt of 266, which was that of the methyl ester of a diunsaturated C_{16}-acid. After catalytic hydrogenation, the GC retention time coincided with that of methyl hexadecanoate. Oxidative degra-

TABLE I

Acids Excreted to the Culture Medium

Designation of gas chromatographic peak, Figures 3 and 4 (methyl ester)	Structure	Relative abundance, %	
Saturated acids			
I	11-Methyldodecanoic acid	0.8	
II	*n*-Tetradecanoic acid	7.7	
III	13-Methyltetradecanoic acid	33.2	
IV	*n*-Pentadecanoic acid	1.9	
V	14-Methylpentadecanoic acid	0.6	
VI	*n*-Hexadecanoic acid	8.3	
VII	15-Methylhexadecanoic acid	15.3	
			67.8
Unsaturated acids			
VIII	*cis,cis*-Tetradec-5,9-dienoic acid	0.1	
IX	*cis*-Tetradec-9-enoic acid	1.5	
X	*cis*-13-Methyltetradec-9-enoic acid	0.8	
XI	*cis,cis*-Hexadec-7,11-dienoic acid	2.1	
XII	*cis*-Hexadec-11-enoic acid	21.4	
XIV	*cis*-15-Methylhexadec-11-enoic acid	5.0	
			30.9
XIII	Unidentified	0.7	
Not designated	Unidentified	0.6	
		100.0	

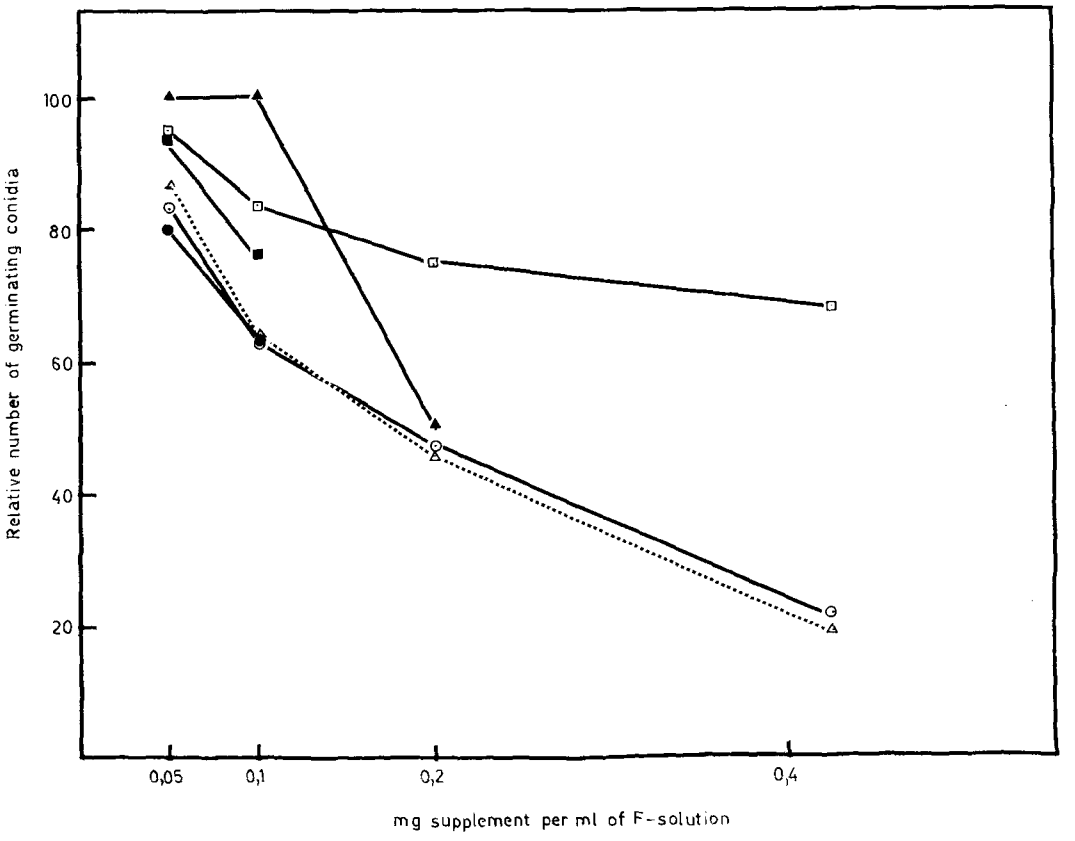

FIG. 8. Relative number of germinating conidia of *F. roseum* after 3 hr incubation at 25 C. Control = 100. Supplement to F-solution: △ synthetic mixture corresponding to the saturated moiety of natural fatty acids, ○ 13-methyltetradecanoic acid, ● *cis*-15-methylhexadec-11-enoic acid, ▲ *cis,cis*-hexadec-7,11-dienoic acid, ■ *cis*-hexadec-11-enoic acid, □ *n*-tetradecanoic acid.

dation, as indicated above, yielded methyl *n*-pentanoate and dimethyl heptan-1,7-dioate accompanied by a small amount of dimethyl succinate. This indicated that the double bonds are situated at positions 7:8 and 11:12. No absorption was observed at 965 cm^{-1} in the IR. Thus, XI must be the methyl ester of *cis,cis*-hexadec-7,11-dienoic acid.

The mass spectrum of component XIV was reproduced in Figure 7. It indicated a mol wt of 282, which was that expected for the methyl ester of a monounsaturated C_{17}-acid. The ions of even mass at m/e 250 (=M-32), m/e 208 (=M-74), and m/e 166 (=M-116), characteristic for long chain monounsaturated methyl esters, were abundant. The mass spectrum differed, however, from the normal chain unsaturated methyl esters, because prominent peaks were observed at m/e M-55 (=227) and m/e M-87 (=195). The former peak was most likely due to loss of a butylene unit from the molecular ion, whereas the latter was due to loss of the $(CH_3O\overset{O}{\overset{\|}{C}}CH_2CH_2-)^+$ ion.

After hydrogenation and subsequent GC examination, it was shown that XIV belonged to the *iso*-series of fatty acids. The products obtained on oxidative degradation showed that the double bond was situated at the 11:12 position. Examination of XIV in the IR indicated *cis* geometry. XIV was, accordingly, the methyl ester of *cis*-15-methylhexadec-11-enoic acid.

The mass spectrum of IX indicated a mol wt of 240. This corresponded to the methyl ester of a monounsaturated C_{14}-acid. In the manner described, the structure was identified as methyl *cis*-tetradec-9-enoate. It was seen from Figure 5 that IX was accompanied by a small amount of a component with a somewhat shorter GC retention time (VIII). MS and GC of the hydrogenated product indicated that VIII was the methyl ester of a normal chain C_{14} diunsaturated acid. It was not possible to

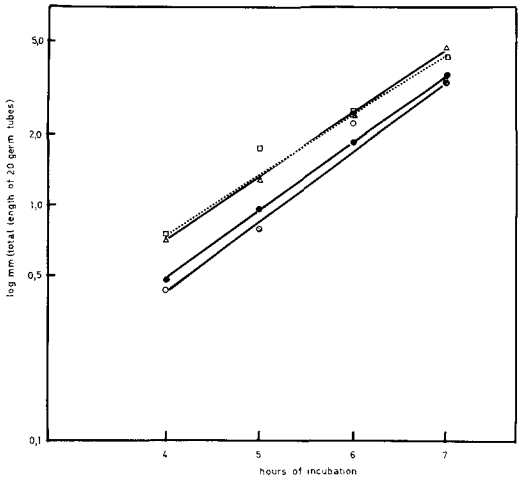

FIG. 9. Growth (total length) of germ tubes, produced by 20 conidia of *F. roseum* at 25 C in F-solution supplemented with: • synthetic mixture corresponding to the saturated moiety of natural fatty acids (0.2 mg/ml), ○ 13-methyltetradecanoic acid (0.2 mg/ml), △ *n*-hexadecanoic acid (0.2 mg/ml), □ non-supplemented F-solution (control).

obtain a sufficient amount of pure VIII for an oxidative degradation experiment. It appears likely that the two double bonds of VIII in analogy with XI were located at the positions 5:6 and 9:10.

The mass spectrum of X indicated a mol wt of 254. This showed that X was the methyl ester of a C_{15}-monounsaturated acid. Degradation experiments and IR examination showed that X possessed the structure of methyl *cis*-13-methyltetradec-9-enoate. The structures, as well as the relative abundance of the components in the inhibitory fatty acid fraction, are summarized in Table I.

Inhibitory effect of the isolated fatty acids on fungi: The biological effect of some lipid fractions and individual fatty acids was examined in germination experiments with conidia of *F. roseum* as the main test material. To facilitate a comparison between different experiments, the germination frequency of the controls was set to 100 and that of the test series was related to this scale. Each point in the diagrams represented the examination of ca. 3000 conidia.

The inhibitory effect of the fatty acid components of the total lipophilic extract of *M. xanthus* and of its saturated and unsaturated moieties was demonstrated in Figure 2. After 3 hr incubation at 25 C, the germination of *Fusarium* conidia was inhibited to the same extent by all three fatty acid fractions. No marked difference in activity between saturated and unsaturated material was observed. Due to

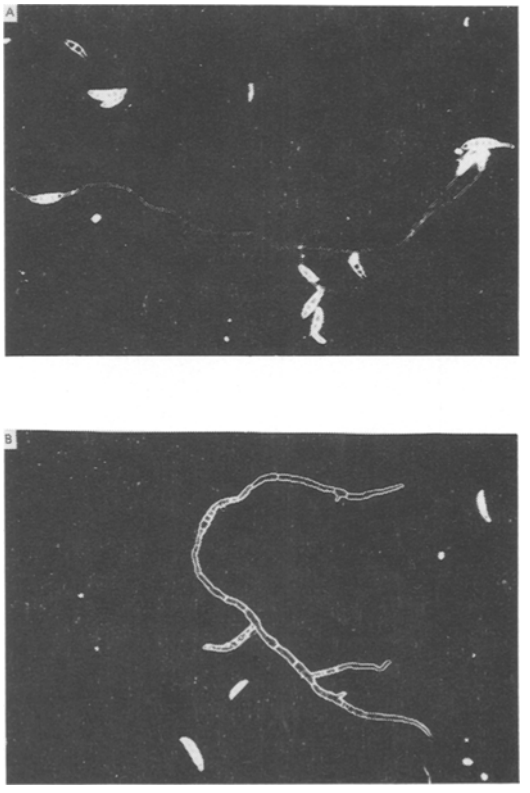

FIG. 10. Hyphae of *F. roseum* grown in F-solution, non-supplemented (a) and supplemented (b) with 0.2 mg/ml F-solution of the natural fatty acid mixture x 400.

the presence of an inhibitory component of nonfatty acid type (fraction 3, Fig. 1), the total lipophilic extract of the culture solution gave a stronger inhibition than the fatty acid mixtures. Clearly, no inhibition was produced by the neutral moiety of fraction 1.

In Figure 8, the inhibitory effect of some of the isolated fatty acids was compared with that of a mixture of synthetic saturated fatty acids. The mixture and the main component of the natural acids, 13-methyltetradecanoic acid, exhibited the same inhibitory effect on conidia germination. Also the branched unsaturated C_{17} acid was markedly inhibitory. In contrast, the straight chain saturated and monounsaturated acids affected the germination to a considerably less degree. In the case of the diunsaturated C_{16} acid, a marked inhibition was observed. Furthermore, tests with 12-methyltridecanoic acid, which was not a natural constituent, showed that this acid acted essentially in the same way as the natural next higher homolog. The results obtained indicated that the fatty acids with *iso*-configuration had an effect on the conidia germination markedly

different from that of the corresponding straight chain structures. The question of saturation or unsaturation appeared to be of minor importance.

Figures 2 and 8 demonstrated the inhibitory effects on the germination of *Fusarium* conidia observed after 3 hr incubation. However, the inhibitions produced by the fatty acids almost were overcome when the incubation period was prolonged to 4 hr. Thus, the natural fatty acid mixture and its active components acted as fungistatic agents.

In another study the fatty acids were investigated with respect to their effect on the growth of the germ tubes. The development of germ tubes produced by 20 randomly selected *Fusarium* conidia in each series was followed by measurements in the microscope during 7 hr (Fig. 9). In the media containing the saturated fatty acid mixture or 13-methyltetradecanoic acid, the germination process was delayed. The development in these series appeared about 1 hr behind that of the control. However, after the germination was over, the germ tubes grew at the same rate in all series.

The fungistatic character of the inhibitory fatty acids also had been established in a number of growth experiments where the development was followed during 15 days. In media supplemented with the relevant fatty acids, used separately or in mixtures and in various amounts, the primary effect was consistently a delayed spore germination, while the subsequent mycelial growth in the test series and control was parallel.

DISCUSSION

Quite a large number of bacteria have been investigated with respect to their lipid content. In particular, the free and bound fatty acids have been analyzed (12). It now has been found that not less than 55% of the free fatty acids excreted by *M. xanthus* possess methyl-branched carbon skeletons. The ability to synthesize such fatty acids sometimes is considered specific to bacteria. It seems, nevertheless, that methyl-branched acids have been observed in only a restricted number of species. In case of the old works, "a reinvestigation of the fatty acid composition is needed with the finer methods available today" (12).

In practically all investigations dealing with lipids of bacteria, the material has been extracted from the bacterial cells. The knowledge of the chemical composition of the excretions to the surroundings by the organisms themselves is consequently very deficient. Since a marked fungistatic action of excreted *iso*-fatty acids has been clearly demonstrated in the case of *M. xanthus,* it appears desirable to investigate the chemical nature of excreted lipids of other soilborne bacteria.

It is not the object of this paper to discuss the mode of action of the *iso*-fatty acids. However, it may be mentioned that the inhibitory fatty acids investigated produced marked morphological changes of both the *Fusarium* conidia and the hyphae developed. The conidia swelled and the hyphae produced were of a short and thick appearance (Fig. 10). It seems very likely that the *iso*-fatty acids act on the plasma membrane. Studies of the mechanism of action will be published elsewhere.

ACKNOWLEDGMENTS

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REFERENCES

1. Dworkin, M., Ann. Rev. Microbiol. 20:75 (1966).
2. Norén, B., Physiol. Plant., In press.
3. Schröder, J., and H. Reichenbach, Arch. Mikrobiol. 71:384 (1970).
4. Ställberg-Stenhagen, S., and E. Stenhagen, in "Topics in Organic Mass Spectrometry," Edited by A.L. Burlingame, Interscience Press, New York, 1970, Chapter 5.
5. Milburn, A.H., and E.V. Truten, J. Chem. Soc. 3344 (1954).
6. Hunsdiecker, H., and C. Hunsdiecker, Chem. Ber. 75:291 (1942).
7. Weitzel, G., and J. Wojahn, Z. Physiol. Chem. 287:65 (1951).
8. Bellamy, L.J., in "The Infra-red Spectra of Complex Molecules," Methuen & Co. Ltd., London, 1958, Chapter 3.
9. de Vries, B., Chem. Ind. (London) 1049 (1962).
10. de Vries, B., JAOCS 40:184 (1963).
11. Odham, G., and E. Stenhagen, in "Biochemical Applications of Mass Spectrometry," Edited by G. Waller, Wiley/Interscience Publishers, 1972, Chapter 8.
12. Asselineau, J., in "The Bacterial Lipids," Hermann, Paris, 1966, p. 206.

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Cardiac Fatty Acids in Rats Fed Marine Oils

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ABSTRACT

Cardiac fatty acids were studied in young rats fed marine oils for 1 week. When the diet contained 0, 0.5, 1, 2, 4, 8 or 16% by weight of partially hydrogenated oil from Norwegian capelin, the concentration of fatty acids in the cardiac tissue was elevated only at the highest level. The amount of the lipid and the content of docosenoic acid in the heart were less than those observed with 15% partially hydrogenated oil from Canadian herring. Nonhydrogenated Peruvian anchovy oil lacking docosenoic acid produced no change in the amount of fat deposited. The extent of fatty acid accumulation in the heart was related to the dietary C_{22} acids.

INTRODUCTION

The C_{22} polyenoic acids of marine oils largely are converted during partial hydrogenation to a mixture of positional and geometric monoenoic isomers (1). When oils containing appreciable quantities of docosenoic acids were fed to rats for periods up to a week, fatty acids in the form of triglycerides accumulated in the myocardium (2-5). To determine the effects of cardiac fatty acids of marine oils, which differ in their content of C_{22} acids, partially hydrogenated Norwegian capelin oil, partially hydrogenated Canadian herring oil, and nonhydrogenated Peruvian anchovy oil were fed to rats.

TABLE I

Fatty Acids[a] of Partially Hydrogenated Capelin Oil (HCO), Partially Hydrogenated Herring Oil (HHO) and Liquid Anchovy Oil (AO)

Fatty acid	HCO	HHO	AO
14:0	7.6	6.6	9.0
15:0	0.7	0.2	0.2
16:0	15.4	11.1	20.5
16:1	8.7	7.2	13.4
17:0	0.8	0.5	1.9
18:0	4.0	2.2	3.2
18:1	14.9	13.4	19.1
18:2	2.3	1.4	1.4
20:0	2.5	1.8	0.2
20:1	14.9	20.5	2.2
20:poly	6.7	0.1	20.0
22:0	1.9	2.9	
22:1	14.3	31.3	0.7
22:poly	4.4	0.5	8.7
Total *trans*	62.7	27.9	

[a]%, by weight. Minor amounts of other fatty acids were detected.

METHODS

Male weanling rats from the Charles River Breeding Laboratories, Wilmington, Mass., were fed test fats or oils as 20% (by weight) of a purified diet (5) for 7 days. The marine oils differed in their content of C_{22} fatty acids (Table I). In one experiment partially hydrogenated capelin oil from Norway (mp 31-33 C) was fed as 0, 0.5, 1, 2, 4, 8 or 16% with 4% corn oil and sufficient lard to make all diets 20% fat. In another experiment, partially hydrogenated Canadian herring oil and liquid Peruvian anchovy oil each were fed at the 15% level with 5% corn oil in the diet. The control fat was a 3:1 mixture of lard and corn oil.

Cardiac lipids were extracted and methyl esters of fatty acids prepared as previously described (3). Each sample was chromato-

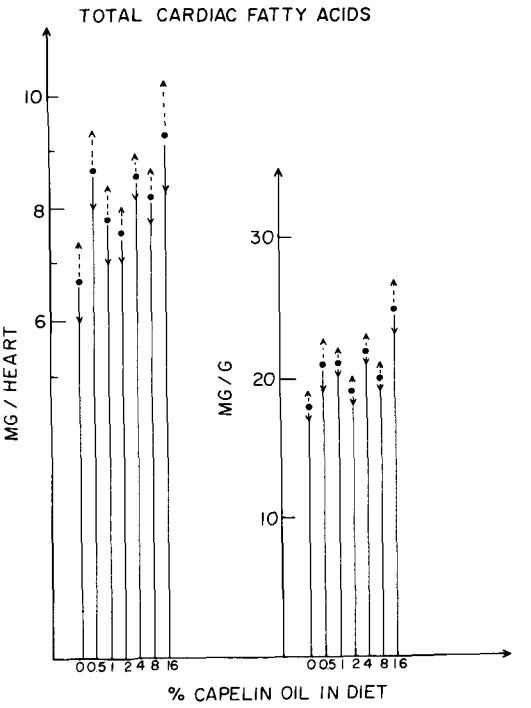

FIG. 1. Cardiac fatty acids of rats fed different levels of partially hydrogenated capelin oil in a 20% fat diet for 1 week. Mean ± standard error of the mean for 5 to 6 determinations for each group is indicated.

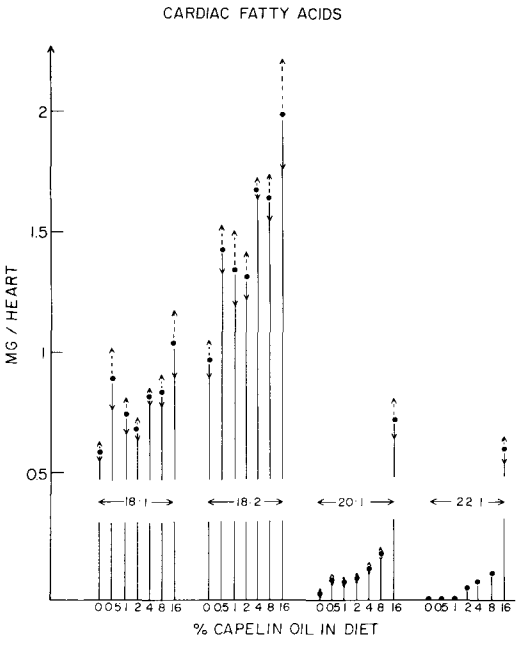

FIG. 2. Octadecenoic acid, octadecadienoic acid, eicosenoic acid, and docosenoic acid of cardiac fatty acids from rats fed different levels of partially hydrogenated capelin oil.

graphed on 2 different columns in a 4000 series Victoreen instrument. With 6 ft, 1/8 in. OD column of 10% butanediol succinate on Anachrom ABS, 80/90 mesh, long low peaks were obtained for the methyl esters of C_{22} polyenoic acids. With a 10% silicone column, programed from 200-300 C at 7.5 C/min, sharp peaks but poor separations were obtained for these components. The data from the silicone column were used to calculate the proportion of each chain length; the data from the polyester column were used to calculate the proportion of each degree of unsaturation. Methyl lignocerate was the internal standard used for quantitation. The signal from the hydrogen-flame detector was amplified, converted to a frequency modulated signal for recording on a Sony tape deck, and later demodulated by the Dallas Instrument recording system, model SY-26-1, for integration on a Hewlett-Packard instrument 3370A at 2 or 4 times the original recording speed. The accelerated rate of replay improved the definition of the C_{22} peaks from the polyester column.

RESULTS

The amounts of total fatty acids in the hearts of rats fed each level of capelin oil are shown in Figure 1. When the data were expressed as mg fatty acids/g heart, intermediate levels of the dietary oil were associated with amounts of cardiac fatty acids which did not differ significantly from the controls, but the highest level of capelin oil produced a significantly increased concentration of fatty acids ($P=0.01$). The results for cardiac 18:1, 18:2, 20:1, and 22:1 fatty acids are shown in Figure 2. With increasing amounts of capelin oil in the diet, the tissue contained increasing amounts of these fatty acids. No change in the amounts of saturated or other polyenoic acids was observed.

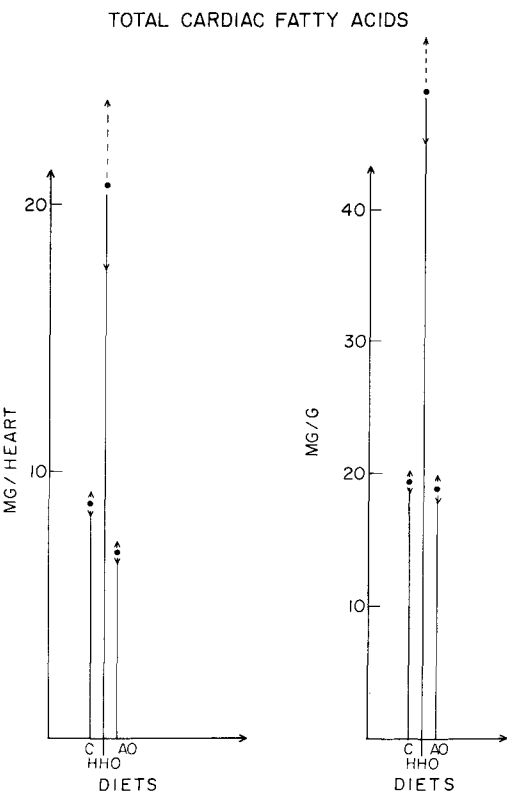

FIG. 3. Cardiac fatty acids of rats fed the control fat mixture (C) consisting of 15% lard and 5% corn oil, 15% partially hydrogenated herring oil (HHO) and 5% corn oil, or 15% liquid anchovy oil (AO) and 5% corn oil.

In the other experiment, the deposition of cardiac fatty acids was increased greatly by the partially hydrogenated herring oil, but not by the liquid anchovy oil (Fig. 3). Specifically the 18:1, 18:2, 20:1, and 22:1 fatty acids increased in the hearts of rats fed the herring oil (Fig. 4). With the anchovy oil there was no evidence of cardiac 20:1 or 22:1 fatty acids.

DISCUSSION

The selection of a 1 week period for the

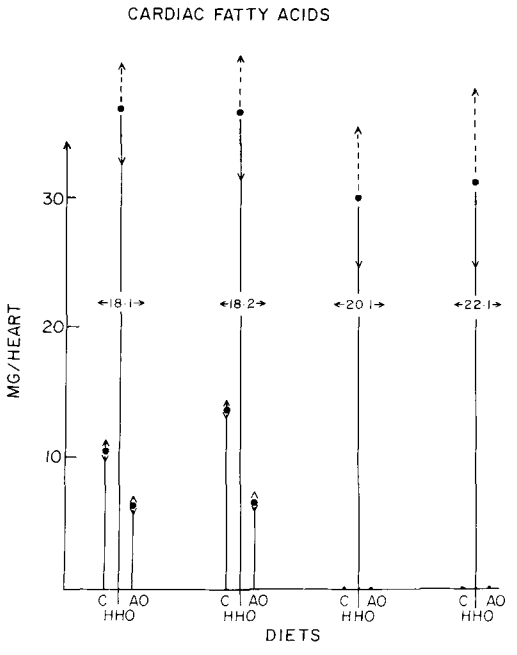

FIG. 4. Octadecenoic acid, octadecadienoic acid, eicosenoic acid and docosenoic acid of the cardiac fatty acids from rats fed the control diet (C), partially hydrogenated herring oil (HHO) or liquid anchovy oil (AO).

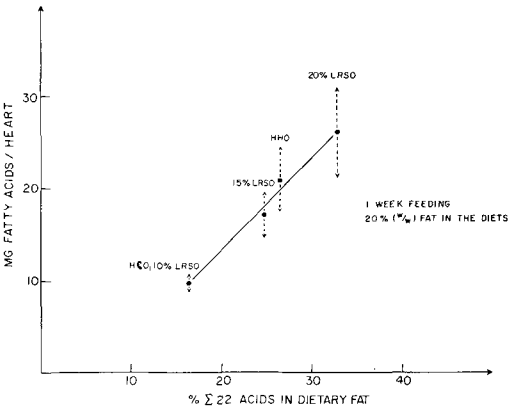

FIG. 5. Relationships between proportion of total C_{22} fatty acids in the dietary fatty acids and amount of cardiac fatty acids. LRSO = liquid rapeseed oil; HHO = hydrogenated herring oil; HCO = hydrogenated capelin oil.

study of maximum lipid deposition in the heart was based on previous work (3). This early interval was not examined by Odense and Brockerhoff (6) who reported no effect on cardiac tissue of partially hydrogenated herring oil after 24 days. Docosenoic acids from marine sources, fed at high levels for 1 week, have been shown to produce an increased accumulation of fatty acids in the heart (3,5,7). As demonstrated in the present study, when the dietary intake of these long chain fatty acids was ca. 3% by weight of the diet (6% of calories in the diet used), the amount of cardiac lipids was increased significantly.

Partially hydrogenated capelin oil fed at the 16% level (as described) and liquid rapeseed oil diluted to 10% in a 20% fat diet (3) provided similar proportions of C_{22} fatty acids in the diet and produced a similar deposition of cardiac fatty acids. Increasing the amount of these long chain fatty acids in the diet, either by rapeseed or herring oil, increased the accumulation of lipid in the heart. A linear relationship between the proportion of C_{22} fatty acids in the diet and the amount of fatty acids deposited in the heart was observed (Fig. 5). In the case of the liquid rapeseed oil, the C_{22} acids were mostly erucic acid (22:1Δ13), whereas the partially hydrogenated marine oils contained a mixture of geometric and positional isomers. These have been studied extensively in hydrogenated herring oil (8,9). The docosenoic isomers found in cardiac lipids were similar to those observed in the dietary oil (10).

Since most of the C_{22} fatty acids in both the capelin and herring oils were monoenoic, it was not possible to ascertain the effects of C_{22} polyenoic acids. These represented most of the C_{22} acids in the anchovy oil but were in sufficiently low concentration that, even if they did produce an effect similar to that of the monoenoic acids, they would probably not have augmented significantly the amount of cardiac fatty acids as determined by this procedure. In such an oil the influence of a high level of C_{20} monoenoic acid has yet to be assessed.

The amounts of monoenoic acids were elevated particularly in the accumulated cardiac lipids. It was demonstrated previously that little deposition of lipid occurred with a high dietary intake of 20:1 fatty acid in the absence of 22:1 (7). In rats fed high levels of capelin oil, and therefore low levels of 18:1, the amount of cardiac 18:1 fatty acid was elevated. Its accumulation in the heart could have resulted from retention of this acid during the lipidosis and from partial β-oxidation of the docosenoic acids (11,12). An increase in the level of cardiac 18:2 fatty acid was found previously in conjunction with a high level of dietary linolenate (13). In the present study with capelin and herring oils, an accumulation of 18:2 also occurred.

The extent of cardiac lipidosis of the young rat fed marine oil depended primarily upon the intake of docosenoic acids.

ACKNOWLEDGMENTS

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REFERENCES

1. Lambertsen, G., H. Myklestad and O.R. Braekkan, JAOCS 48:389 (1971).
2. Abdellatif, A.M.M., and R.O. Vles, Nutr. Met. 12:285 (1970).
3. Beare-Rogers, J.L., E.A. Nera and H.A. Heggtveit, Can. Inst. Food Technol. J. 4:120 (1971).
4. Rocquelin, G., J.P. Sergiel, B. Martin, J. Leclerc and R. Cluzan, JAOCS 48:728 (1971).
5. Beare-Rogers, J.L., E.A. Nera and B.M. Craig, Lipids 7:548 (1972).
6. Odense, P.H., and H. Brockerhoff, J. Fish. Res. Bd. Can. 28:1793 (1971).
7. Beare-Rogers, J.L., E.A. Nera and B.M. Craig, Lipids 7:46 (1972).
8. Ackman, R.G., S.N. Hooper and J. Hingley, JAOCS 48:804 (1971).
9. Conacher, H.B.S., B.D. Page and R.K. Chadha, Ibid. 49:520 (1972).
10. Conacher, H.B.S., B.D. Page and J.L. Beare-Rogers, Lipids 8:256 (1973).
11. Craig, B.M., and J.L. Beare, Can. J. Biochem. 45:1075 (1967).
12. Carreau, J.P., A. Thoron, D. Lapous and J. Raulin, Bull. Soc. Chim. Biol. 1973 (1968).
13. Mohrhauer, H., and R.J. Holman, J. Nutr. 81:67 (1963).

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Incorporation of Choline-1,2-^{14}C into Molecular Species of Phosphatidylcholine by Alfalfa Leaflet Tissue[1]

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ABSTRACT

Alfalfa leaflet tissue incorporated choline-1,2-^{14}C into at least 6 molecular species of phosphatidylcholine. After 7 hr incubation the species containing palmitic and linoleic acids (16:0-18:2) and the species containing linolenic and linoleic acids (18:3-18:2) had the highest specific activities. After 24 hr incubation, the species containing palmitic and linolenic acids (16:0-18:3) and the species containing 2 linolenic acids (18:3-18:3) had the highest specific activities. The most likely interpretation of these data is that choline is accepted by diglycerides containing one linoleic acid residue and that this residue is then desaturated within the phosphatidylcholine molecule to linolenic acid.

INTRODUCTION

Generally it is accepted that the substrate of fatty acid desaturases are either acylCoA or acyl carrier protein (ACP) derivatives (1-4). However, complex glycerides have been implicated in the desaturation of fatty acids in algae (5-10) and in higher plants (11). The main lipids involved were monogalactosyl diglyceride (MGDG) and phosphatidylcholine (PC). This conclusion had been reached by labeling the fatty acids with ^{14}C-labeled precursors.

Evidence presented in this paper for the involvement of PC in the desaturation of linoleic acid to linolenic acid in alfalfa leaflet tissue is based on the labeling of molecular species of PC with choline-1,2-^{14}C.

PROCEDURES

Incubation with choline-1,2-^{14}C: Young alfalfa (*Medicago media*, var. Rambler) leaflets from 3-week-old seedlings were cut into pieces with a razor blade and shaken under semisterile conditions at 23 C in 125 ml Erlenmeyer flasks containing 5 μc choline-1,2-^{14}C (New England Nuclear, 5.8 mc/mM) in 6 ml 0.02 M phosphate buffer at pH 5 and 1 g tissue. After 7, 16, and 24 hr incubation, tissue was rinsed repeatedly with distilled water, kept for 3 min in boiling water, again rinsed repeatedly with water and finally covered with 15 ml chloroform-methanol (2:1, v/v).

Extraction and purification of lipids: The tissue was homogenized in chloroform-methanol (2:1, v/v). The homogenate was filtered, and the residue was extracted twice more with the same solvent. The water solubles were removed from the extract by the method of Williams and Merrilees (12) using Sephadex G 25 medium. The purified extract finally was taken up in a small volume of chloroform-methanol (2:1, v/v).

Separation of lipids: The lipids were separated by thin layer chromatography (TLC) in three steps: (a) separation of phospholipids from glycolipids, neutral lipids, and pigments on Silica Gel G in acetone-acetic acid-water (100:2:1, v/v) (13), (b) separation of PC from other phospholipids on Silica Gel G in chloroform-methanol-acetic acid-water (85:12.5:12.5:2, v/v) (14), and (c) separation of the molecular species of PC on silver nitrate impregnated Silica Gel H (20% $AgNO_3$ in silica, w/w) in chloroform-methanol-water (65:25:4, v/v) (15). The lipids were detected by spraying with a 0.005% Rhodamine 6 G aqueous solution and viewing under UV light. The molecular species of PC were characterized further by spraying the TLC plate with the reagent of Dittmer and Lester for phosphorus (16) and a modified Dragendorff's reagent for choline (17). The bands were eluted from the plates with the following solvents used in sequence: chloroform-methanol (2:1, v/v), chloroform-methanol-acetic acid-water (50:40:10:1, v/v), methanol-water (1:1, v/v), and methanol.

Fatty acid analysis: The fatty acids of the PC bands were analyzed by gas liquid chromatography (GLC) after transesterification in methanolic borontrifluoride (18).

Characterization of radioactive moiety in PC: Purified labeled PC was hydrolyzed completely by refluxing in 3 N HCl at 100 C for 2 hr. Fatty acids were extracted from the hydrolysate with petroleum ether, and radioactivity of both fractions was determined. Water soluble hydrolysis products were cochromatographed on a thin layer of Silica Gel G in butanol-NH_4OH (10:90, v/v) with added choline and glycerol. Choline was detected with I_2 vapor; radioactivity was located on the plate by means

[1]Contribution No. 39, C.D.A. Research Station, Ste-Foy, Quebec.

of a scanner (Actigraph II, Nuclear Chicago); glycerol was then detected with 5% potassium dichromate in 40% sulfuric acid.

Measurement of radioactivity: Radioactivity was assayed by scintillation counting (Unilux II, Nuclear Chicago) using Bray's solution (19). Data were corrected for quenching by the external standard ratio method. Radioactivity of the molecular species of PC was measured directly on the plates by means of the scanner. Radioautography of TLC plates was made using Kodak X-ray No Screen film.

RESULTS AND DISCUSSION

Choline-1,2-^{14}C is readily incorporated into the lipids of alfalfa leaflet tissue (Table I). Radioactivity (50-75%) incorporated into the lipids is recovered in PC after 2 TLC purification steps, in which the phospholipid band and then the PC band are eluted from the plate.

After hydrolysis of the labeled PC, no radioactivity was detected in the petroleum ether fraction, i.e. the fatty acid moiety. All water soluble radioactivity was cochromatographed with choline, none with glycerol.

After separation of the molecular species of PC according to their degree of unsaturation, at least 8 bands were detected with Rhodamine 6G. Of these all but the 2 more saturated ones, i.e. the 2 bands moving nearest the solvent front, were labeled (Fig. 1). Bands 1-5 and 7-8 gave a positive reaction with the reagent of Dittmer and Lester for phosphorus and with Dragendorff's reagent for choline. The radioautogram of the TLC plate shows a progressive relative increase of the labeling in bands 1 and 3.

Radioactivity incorporated in the molecular species at different sampling times is given in Table II, both in dpm/g fresh wt tissue and in percentage of the radioactivity incorporated in PC. Numbers of the species refer to the numbers by which they were identified in Figure 1. The data confirm that there is a relative increase in radioactivity in species 1 and 3, with a simultaneous relative decrease in radioactivity in species 2 and 5, thus a tendency toward more desaturation with longer times of incubation.

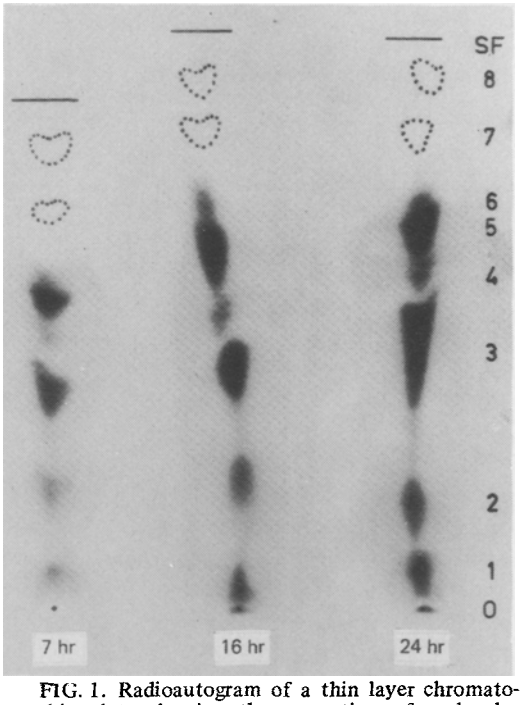

FIG. 1. Radioautogram of a thin layer chromatographic plate showing the separation of molecular species of phosphatidyl choline according to their degree of unsaturation.

Fatty acid composition of the species was determined on an unlabeled sample to which radioactive PC had been added as a marker (Table III). From these data the labeled molecular species can be identified readily and their purity evaluated. Species 1 (hexaenes) is predominantly dilinolenyl-PC (18:3-18:3). Species 2 (pentaenes) is almost pure linolenyl-linoleyl-PC (18:3-18:2). Species 3 (trienes) is a mixture of mainly palmityl-linolenyl-PC (16:0-18:3), with some stearyl-linolenyl-PC (18:0-18:3) and some oleyl-linoleyl-PC (18:1-18:2). Species 4 (tetraenes) is almost pure dilinoleyl-PC (18:2-18:2). Species 5 and 6 (dienes) are predominantly palmityl-linoleyl-PC (16:0-18:2) with significant amounts of stearyl-linoleyl-PC

TABLE I

Incorporation of Choline-1,2-^{14}C into Lipids, Phospholipids, and Phosphatidylcholine[a]

Time of sampling (hr)	Total	Phospholipids	Phosphatidylcholine
7	12	10	9
16	21	15	11
24	26	23	18

[a]Data expressed as percentage of radioactivity initially present in incubation medium.

TABLE II

Radioactivity in Molecular Species of Phosphatidylcholine after 7, 16 and 24 hr of Incubation

Species		Time of sampling 7 hr	16 hr	24 hr
1	dpm[a]	18,218	29,242	55,504
	%[b]	9.4	11.7	13.8
2	dpm	41,237	45,280	52,890
	%	21.3	18.0	13.2
3	dpm	60,170	87,725	181,915
	%	31.1	35.0	45.2
4+5+6	dpm	73,955	88,754	111,932
	%	38.2	35.4	27.8
Total dpm		193,600	251,000	402,200

[a]dpm/g fresh wt of tissue.

[b]Percentage of total radioactivity in PC.

(18:0-18:2) and dioleyl-PC (18:1-18:1). Why tetraenes migrate faster than trienes in this system is not known, but species containing 2 diene fatty acids possibly react less with silver nitrate than species containing 1 triene. Indeed, Gurr and Brawn (7) have observed that species (18:1-18:3) and (18:0-18:2) migrate more slowly than species (18:2-18:2) and (18:1-18:1) respectively.

There is no radioactivity associated with molecular species containing less than 2 double bonds, although 2 such species, bands 7 and 8, have been detected in low amounts on the TLC plates. This suggests specificity with respect to the fatty acid composition of the choline accepting molecules, either diglycerides if incorporation is via the Kennedy pathway involving CDP-choline (20) or phospholipids if incorporation is via exchange reactions mediated by phospholipase D (21).

The approximate specific activity of the molecular species has been calculated from the data of Tables II and III (Table IV); the dpm of each band was calculated from the total dpm in PC and from the percentage of this radioactivity in each band as determined by means of the scanner. The mass of each band was calculated from the GLC analyses, assuming as average mol wt of the 2 fatty acids of the PC molecules that of stearic acid (18:0). Species 4 and 6 have little mass and radioactivity as compared with species 5. The specific activity calculated from the data for the group of species 4, 5, and 6 combined should, therefore, approximate the specific activity of species 5. The table shows that after 7 hr incubation, the 2 species with higher specific activities are species 2, which contains linolenyl and linoleyl residues (18:3-18:2), and species 5, palmityl-linoleyl-PC (16:0-18:2). However, the specific activities of species 1 (18:3-18:3) and species 3 (16:0-18:3) increase rapidly, and after 24 hr incubation their specific activities are markedly higher than the specific activities of species 2 and 5.

The most likely interpretation of these data is that choline is preferentially accepted by species of diglycerides or phospholipids which contain 1 linoleyl residue and that this residue is then further desaturated within the PC molecules.

Specificity at the level of the accepting diglycerides has been shown already in animal

TABLE III

Fatty Acid Composition of the Molecular Species of Phosphatidylcholine

Species		Fatty acids[a] 16:0	16:1	18:0	18:1	18:2	18:3	Total
1	%	7	4	1	2	3	82	
	μg[b]	1.9	1.1	0.3	0.6	0.7	20.7	25.3
2	%	2				50	48	
	μg	0.5				16.6	15.7	32.8
3	%	37		9	3	2	49	
	μg	30.3		7.6	2.2	1.6	40.1	81.8
4	%	7				93		
	μg	1.2				16.0		17.2
5+6	%	37		8	10	45		
	μg	28.7		5.9	7.9	35.1		77.6
7[c]	%	37	25	15	24			
	μg	2.6	1.7	0.9	1.7			6.9
8[c]	%	44	24	18	14			
	μg	3.7	2.0	1.5	1.2			8.4

[a]Length of carbon chain:number of double bonds.

[b]μg per gram fresh wt of tissue.

[c]Fatty acids determination for bands 7 and 8 were made on a separate sample.

tissues. Indeed Rytter and Cornatzer (22) observed that choline-1,2-^{14}C was incorporated mainly in PC species containing 1 or 2 double bonds, while ethanolamine-1,2-^{14}C was incorporated into polyunsaturated PC species via methylation of phosphatidylethanolamine.

Biosynthesis of polyunsaturated species of PC by methylation was shown also by Tinoco et al. (23). However, lack of specificity of choline phosphotransferase toward diglycerides was observed by Devor and Mudd (24) in spinach leaves and by Mudd et al. (25) in animal tissue. In spinach leaves saturated species of diglycerides, nevertheless, failed to stimulate choline incorporation, while diolein gave only slight stimulation (24). Devor and Mudd believe that in spinach leaves fatty acid distribution in PC is not controlled by choline phosphotransferase but by acyltransferase (26). Mudd et al. (27) have shown that, in the case of galactolipid biosynthesis in spinach leaves, there was marked diglyceride specificity. Data show that in alfalfa leaves there is a strong preference for the incorporation of choline-1,2-^{14}C into PC species (18:3-18:2) and (16:0-18:2).

The progressive shift of the radioactivity of choline-1,2-^{14}C from species containing a linoleyl residue to the corresponding species containing a linolenyl residue supports the suggestion by James, Nichols, Gurr, and their coworkers (5-10) that some fatty acid desaturases require PC as their substrate and also the suggestion by Roughan (11) that the primary site of linolenic acid biosynthesis in leaf cells is within the PC molecule. Direct desaturation of the phospholipid would appear to be the most efficient mechanism (28). Another possibility, which Hitchcock and Nichols (29) consider more likely, is that the fatty acid is transferred from the lipid to the desaturase enzyme and back to the lipid after desaturation.

TABLE IV

Approximate Specific Activity (μc/mM) of Molecular Species of Phosphatidylcholine after 7, 16, and 24 hr Incubation

Bands	Main species	Time of sampling 7 hr	16 hr	24 hr
1	(18:3-18:3)	186	300	568
2	(18:3-18:2)	321	352	410
3	(16:0-18:3)	157	274	569
4+5+6	(16:0-18:2)	202	239	302

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REFERENCES

1. Harris, R.V., and A.T. James, Acta Biochim. Biophys. 106:456 (1965).
2. Kanangara, C.G., and P.K. Stumpf, Arch. Biochem. Biophys. 148:414 (1972).
3. Nagai, J., and K. Bloch, J. Biol. Chem. 241:1925 (1966).
4. Vijay, J.K., and P.K. Stumpf, Ibid. 246:2910 (1971).
5. Appleby, R.S., R. Safford and B.W. Nichols, Acta Biochim. Biophys. 248:205 (1971).
6. Gurr, M.I., Lipids 6:266 (1971).
7. Gurr, M.I., and P. Brawn, Eur. J. Biochem. 17:19 (1970).
8. Gurr, M.I., M.P. Robinson and A.T. James, Ibid. 9:70 (1969).
9. Nichols, B.W., Lipids 3:354 (1968).
10. Nichols, B.W., and R. Moorhouse, Ibid. 4:311 (1969).
11. Roughan, P.G., Biochem. J. 117:1 (1970).
12. Williams, J.P., and H.T. Merrilees, Lipids 5:367 (1970).
13. Gardner, H.W., J. Lipid Res. 9:139 (1968).
14. Kupier, P.J.C., Plant Physiol. 45:684 (1970).
15. Arvidson, G.A.E., J. Lipid Res. 6:574 (1965).
16. Dittmer, J.C., and R.L. Lester, Ibid. 5:126 (1964).
17. Bregoff, H.M., E. Roberts and C.C. Delwiche, J. Biol. Chem. 205:565 (1953).
18. Grenier, G., A. Tremolieres, H.P. Therrien and C. Willemot, Can. J. Bot. 50:1681 (1972).
19. Bray, G.A., Anal. Biochem. 1:279 (1960).
20. Kennedy, E.P., J. Biol. Chem. 209:525 (1954).
21. Vandor, S.L., and K.E. Richardson, Can. J. Biochem. 46:1309 (1968).
22. Rytter, D.J., and W.E. Cornatzer, Lipids 7:142 (1972).
23. Tinoco, J., S.M. Hopkins, D.J. McIntosh, G. Sheehan and R.L. Lyman, Ibid. 2:479 (1967).
24. Devor, K.S., and J.B. Mudd, J. Lipid Res. 12:403 (1971).
25. Mudd, J.B., L.M.G. van Golde and L.L.M. van Deenen, Acta Biochim. Biophys. 176:547 (1969).
26. Devor, K.A., and J.B. Mudd, J. Lipid Res. 12:412 (1971).
27. Mudd, J.B., H.H.D.M. van Vliet and L.L.M. van Deenen, Ibid. 10:623 (1969).
28. Kates, M., and M. Paradis, Can. J. Biochem. 51:184 (1973).
29. Hitchcock, C., and B.W. Nichols, in "Plant Lipid Biochemistry," Academic Press, New York, 1971, p. 145.

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SHORT COMMUNICATION

Stability of Prostaglandin E Compounds in Solution

ABSTRACT

In the present paper the stability of prostaglandins E_1, E_2, and E_3 has been studied in two types of solvents: (a) solvents in which they can be used in clinical trials and (b) solvents which are commonly used for their isolation from biological systems. The solvents used in the first type were ethanol, saline, and water while those in the second type were chloroform and ethylacetate. In addition to this the effect of light on stability of these prostaglandins in chloroform also has been studied.

INTRODUCTION

Ever since various prostaglandins were isolated from human seminal plasma and their structures established (1), they have been used at an increasingly rapid rate in clinical trials both with animals and humans. Known prostaglandins are added as carriers to incubation medium prior to extraction of labeled prostaglandins and their metabolites. This is followed by their resolution by various chromatographic processes. As most prostaglandins are sensitive to the pH of the medium and also to the duration they are in solution, it was studied if the solvent system, duration of storage of the prostaglandin solution, and temperature had any effect on their stability. The solvent system chosen for this purpose are mainly of two types: one like ethanol, saline, and water, which are commonly used in clinical trials of prostaglandins, and the other like chloroform and ethylacetate, which are commonly employed for their isolation from the biological system.

MATERIALS AND METHODS

All solvents used for making stock solutions of the prostaglandins as those used in chromatographic separations were of analytical reagent grade (Merck, Darmstadt). Ethanol was adjusted to 95% alcohol content by adding distilled water before it was used for making solutions.

Prostaglandins E_1, E_2, and E_3 were isolated from human seminal plasma according to the method of Hamberg and Samuelsson (1) and Srivastava and Clausen (2).

The absolute quantity of each of the three prostaglandins was determined after subjecting them to thin layer chromatography (TLC) before making stock solutions of these compounds. This was necessary, because of the possible conversion, although small, of the

TABLE I

Stability of PGE Compounds Stored for Variable Periods in Different Solvents at Different Temperatures[a]

		Ethanol				Chloroform				Ethylacetate			
Storage time (months):		1/2	1	3	5	1/2	1	3	5	1/2	1	3	5
E_1	-15 C	+[b]	+	+	96.8	+	+	+	88.3	+	+	+	93.5
	4 C	+	92.3	---	---	+	+	+	86.4	+	+	+	86.4
	R.T.[c]	---[d]	---	---	---	91.4	77.9	---	---	---	74.2	---	---
E_2	-15 C	+	+	+	97.3	+	+	+	90.0	+	+	+	92.3
	4 C	+	95.4	---	---	+	+	+	85.7	+	+	+	88.1
	R.T.	---	---	---	---	92.2	79.4	---	---	---	70.4	---	---
E_3	-15 C	+	+	+	93.5	+	+	+	87.6	+	+	+	90.2
	4 C	+	88.1	---	---	+	+	+	84.7	+	+	+	83.4
	R.T.	---	---	---	---	89.1	74.2	---	---	---	65.6	---	---

[a]Figures % prostaglandin E left from solutions initially containing 100 µg/l ml after variable duration of storage (months).

[b]+ no measurable loss i.e. yield 97-98.5%.

[c]R.T. = Room temperature (+ 22 C).

[d]Not determined.

prostaglandin E (PGE) compounds into the corresponding prostaglandin A (PGA) compounds during TLC and other isolation procedures (3). The stock solutions of the prostaglandins were prepared in ethanol, chloroform, and ethyl acetate in a concentration of 100 μg per 100 μl. The solutions in saline and water had concentrations of 100 and 50 μg per ml respectively. The solutions were kept at three different temperatures: -15 C, 4 C and room temperature (22 C). A portion of the chloroform solution at room temperature also was kept under two different conditions, i.e. one exposed to daylight (only indirect sunlight) and the other to dark, the latter being achieved by covering the container with aluminum foil.

These solutions were examined for their PGE content intermittently (Table I). Since during storage a part of the PGE compound might be converted into the corresponding PGA by dehydration, it was thought desirable to resolve the PGE from any PGA that might have been formed, prior to the assay of the former. This was achieved by TLC on Silica Gel G using a solvent system consisting of ethyl-acetate-iso-octane-acetic acid-water (110:20:10:100, v/v, equilibrated for 1 hr before using the organic phase) (4). The PGE compound was extracted with methanol and measured spectrophotometrically in the presence of alkali (5).

In the case of saline, as well as aqueous solutions of the PGE compounds, the solutions as such were not applied on the TLC plate. Instead, from these solutions, the PGE compounds were extracted with 2 volumes of chloroform thrice after acidification with acetic acid to pH 3.0. The chloroform extracts were pooled together, washed with a minimum quantity of water until free from acetic acid, concentrated under nitrogen to a small volume, and finally resolved by TLC. In all TLC resolutions the zone due to the PGE was revealed by iodine and marked. The plates were exposed to air and the iodine allowed to go off. The PGE compounds were extracted with methanol and assayed as described above.

RESULTS

The ethanolic solutions of PGE_1, PGE_2, and PGE_3 stored at -15 C did not show any measureable loss in their concentration when examined intermittently over 3 months. At the end of the fifth month a loss between 3 to 6% was observed, the highest being for PGE_3. The solutions of PGE_1 and PGE_2 kept at 4 C in ethanol showed a measurable loss when examined at the end of the first month of storage (5 to 12%). The PGE_1 and PGE_2 compounds dissolved in saline were found to be stable even after 15 days storage at -15 C. At 4 C these 2 deteriorated so fast that only ca 58 to 62% remained after 15 day storage period. The PGE_1 and PGE_2 compounds in aqueous solution kept at -15 C were found to be stable up to one month, when ca 95% were left, whereas 40 to 45% deterioration occurred only after 15 days of storage at 4 C.

TABLE II

Effect of Daylight, Indirect Sunlight, on the Stability of Prostaglandin E Compounds Stored in Chloroform Solution at Room Temperature[a]

PGE compound	7 days	15 days	36 days[b]
E_1	3.1	5.3	7.4
E_2	2.9	5.5	8.6
E_3	5.4	9.2	15.8

[a]Figures % degradation of the prostaglandin E compounds exposed to light when compared with those kept in dark under similar conditions (22 C).

[b]Days of exposure to light.

The degradation pattern of the 3 PGE compounds in chloroform and ethylacetate was almost identical under the 3 storage conditions of temperature. Both at -15 C and 4 C there was no change in concentration over 3 months, but degradation was observed when examined at the end of the fifth month (see Table I). At room temperature (22 C), however, degradation was fast, 20 to 25% in chloroform, and 25 to 35% in ethylacetate was degraded during the first month of storage (Table I).

A greater and progressive loss of the PGE compounds in chloroform solution took place when exposed to light at room temperature than when stored in dark at the same temperature. The loss was found to be maximum for the PGE_3 compound and almost the same for the other 2. The results are shown in Table II.

DISCUSSION

From the degradation pattern of the PGE compounds, it is clear that, if they are to be used in clinical trials, they should be used as alcoholic solution stored at -15C. If they are to be administered in saline, a portion of the concentrated alcoholic solution may be diluted with it to a desired concentration before use.

In the experiments (6) with the biological synthesis of prostaglandins in human platelets, the authors found that from their extraction to column and TLC resolution followed by their assay, it takes several days. The time factor is even more important if one has to deal with platelets from several persons. In such a situation it is best to add 50 μg of each of the

prostaglandin carriers to the chloroform extract, which contains a mixture of prostaglandins and other lipids, and store them at -15C until chromatographic resolution can be taken up. This is possible because of an almost negligible loss of the PGE compounds in chloroform solution at -15 C for several months (Table I).

Literature is now available on extracting prostaglandins from biological systems in connection with various biochemical studies of these compounds; to the authors' knowledge, no one has used antioxidants prior to their extraction. It is not known to what extent naturally occurrring antioxidants protect them from oxidation. Although no attempts have been made here to study the effect of antioxidants on the stability of prostaglandins, it may be possible to enhance the stability in some cases.

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REFERENCES

1. Hamberg, M., and B. Samuelsson, J. Biol. Chem. 241:257 (1966).
2. Srivastava, K.C., and J. Clausen, (Lipids) 8:431 (1973).
3. Hamberg, M. and B. Samuelsson, in "Proceedings of the Second Nobel Symposium," Edited by S. Bergström and B. Samuelsson, Alquist and Wicksell, Stockholm, 1966, p. 63.
4. Bygdeman, M., K. Svanborg, and B. Samuelsson, Clin. Chim. Acta. 26:373 (1969).
5. Nugteren, D.H., R.K. Beerthuis, and D.A. van Dorp, Rec. Trav. Chim. 84:405 (1966).
6. Srivastava, K.C., and J. Clausen, Lipids 7:762 (1972).

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LETTER TO THE EDITOR

Significance of Malonyl-CoA as an Intermediate in Fatty Acid Biosynthesis

Sir: There is a dilemma facing the enzymologist. On one hand, the desire to work with well defined systems means wanting to purify enzymes as much as possible and to keep them at low concentrations (incubations linear with protein and time). On the other hand, the desire to copy nature means changing natural products as little as possible and working under physiological conditions. Early considerations of this dilemmma were presented by Linderstrøm-Lang and Holter (Compt. Rend. Trav. Lab. [Carlsberg] 19: No. 4 [1931]).

In fatty acid biosynthesis there is the question of protein concentrations. Table I lists a series of enzyme concentrations in rat and pigeon livers. The corresponding assay systems for fatty acid synthetase (references as in Table I) contain 2-30 μg enzyme per milliliters. This means that there is an average difference of about a factor 400 between the enzyme concentrations in the natural and in the assay systems.

Guynn et al. (J. Biol. Chem. 247: 7325 [1972]) have recently used enzymatic assays to measure the levels of acetyl-CoA and malonyl-CoA in total rat livers. They find values ranging from 23-114 μM acetyl-CoA and from 4-25 μM malonyl-CoA. These results compare well with the range of substrate concentrations used in the above mentioned model systems for the assay of fatty acid synthetase. However, when seen in relation to the enzyme concentrations listed in Table I, one is struck by a principal difference. In the model systems, substrate concentrations dominate completely over enzyme concentrations; in rat livers, substrate concentrations and especially malonyl-CoA concentrations seem to be nearly equal to enzyme concentrations on a molar basis.

This puts previous considerations of the

TABLE I

Fatty Acid Synthetase Concentrations Calculated from Levels in Particle-Free Supernatant Fraction

		Particle-free supernatant protein		Fatty acid synthetase		
Reference	Sample	mg/ml	Liver, mg/g	Purification factor from particle-free supernatant	Liver, mg/g	Concentration in liver, μM
Burton et al., Arch. Biochem. Biophys. 126:141 (1968)	Rat Liver	40	100	14	7.2	13
Hansen et al., Biochim. Biophys. Acta 210:400 (1970)	Rat Liver	25	63	28[a]	2.2	4
Hsu et al., J. Biol. Chem. 240:3736 (1965)	Pigeon Liver	49	121	21	5.8	13
Burton et al., Arch. Biochem. Biophys. 126:141 (1968)	Pigeon Liver	45[b]	113	14	8.1	18

[a]The specific activities of the particle-free supernatant fractions quoted by Hansen et al. are too low by a factor 2.

[b]It is assumed that Table I in the paper by Burton et al. refers to 40 ml particle-free supernatant for both rat and pigeon livers (cf. Hsu et al., J. Biol. Chem. 240:3736 [1965]).

significance of malonyl-CoA as an intermediate in fatty acid biosynthesis into further perspective. Our suggestion that fatty acid biosynthesis might be based on the direct carboxylation of acetyl groups already bound to fatty acid synthetase, without the formation of an intermediate malonyl-CoA pool (Hansen and Hansen, Acta Chem. Scand. 23: 2180 [1969]; Hansen et al., Biochim. Biophys. Acta 248: 391 [1971]; Carey et al., Ibid. 260: 527 [1972]), agrees with a substrate to enzyme ratio of 1:1. The fact that Guynn et al. (J. Biol. Chem. 247: 7325 [1972]) used enzymatic methods, without identifying CoA derivatives as such, could mean that they were measuring malonyl groups bound to soluble protein from denatured fatty acid synthetase, which were transferable to the excess amount of extra fatty acid synthetase added to the incubation medium for the assay.

Whether or not Guynn et al. (J. Biol. Chem. 247: 7325 [1972]) actually measured malonyl-CoA concentrations, it can in any case be said that the enzyme concentrations presented in Table I are so high, relative to the corresponding substrate concentrations in rat liver, that they warrant the assumption that incubations with excess of ready-made malonyl-CoA may be regarded as biological artifacts. A further elucidation of the malonyl-CoA pathway should include investigations at high enzyme concentrations, disregarding that the incubations may not be proportional to protein.

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ERRATUM

An error occurred in the publication of "Use of a Bile Duct T-Cannula as a New Technique for Studying Bile Acid Turnover in the Rat" by H.C. Klauda, R.F. McGovern and F.W. Quackenbush (Lipids 8:459 [1973]).

On page 462, plate B of Figure 2 was printed upside down. The correct figure and caption appear on the following page.

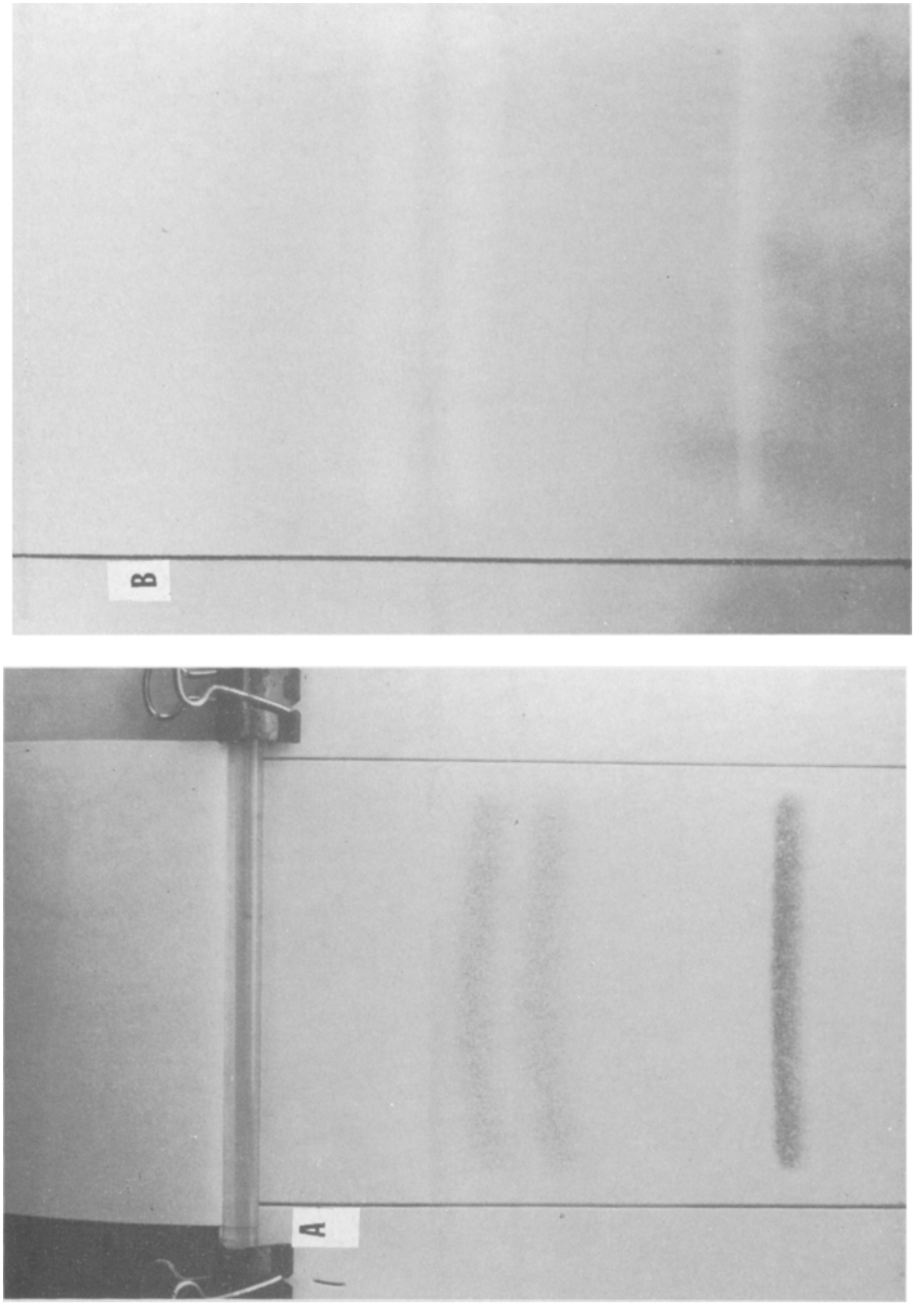

FIG. 2. Relative migration of deoxycholate (DC), chenodeoxycholate (CDC) and cholate (C) when subjected to continuous development thin layer chromatography (TLC). DC, CDC and C (500, 500 and 1000 μg) were applied to 0.5 mm Silica Gel G plates, which were subjected to continuous development TLC using isooctane-ethyl acetate-acetic acid 100:50:14. Developed plates were sprayed with 2% H_2SO_4 in methanol (A) or with water (B) to detect bands. Mobilities relative to DC were 0.82 (CDC) and 0.17 (C).

Analysis of Lipoxygenase Nonvolatile Reaction Products of Linoleic Acid in Aqueous Cereal Suspensions by Urea Extraction and Gas Chromatography

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ABSTRACT

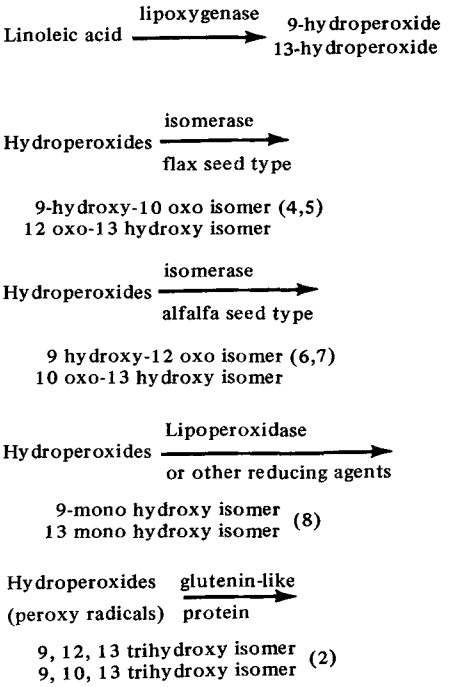
Cereals contain lipoxygenase and small quantities of linoleic and linolenic acids. For studying the oxidation of these acids by lipoxygenase in aqueous cereal suspensions and especially in doughs of wheat flour, an extraction method was developed, providing a means of quantitative extraction of the acids and their oxidation products. A complete extraction can be obtained by using urea as a disclosing agent and a chloroform-isopropanol mixture as the extraction solvent. For the detection of the acids and their oxidation products, some of which are formed in relatively small amounts, a gas chromatographic method was developed. After extraction, the products are first methylated and silylated; then, along with an internal standard, determined with the aid of gas liquid chromatography. This method permits the determination of mono-, di-, and trihydroxy acids, as well as of the ketodihydroxy and α and γ ketols.

INTRODUCTION

The enzyme lipoxygenase (EC 1.13.1.13) forms 2 isomere hydroperoxides or hydroperoxy radicals from linoleic acid added to wheat flour/water suspensions and doughs, viz. the 9-isomer (85%) and the 13-isomer (15%). These hydroperoxides or hydroperoxy radicals are enzymatically converted into different secondary oxidation products. Beside the monohydroxy acids hydroxyepoxy acids also are formed which are converted rapidly into trihydroxy acids (1).

In a study of suspensions of barley flour in water, it was found (with the aid of thin layer chromatography [TLC]) that, in addition to mono and trihydroxy acids, small amounts of dihydroxy and ketodihydroxy acids and α and γ ketols are formed (2). At the same time the same products, though in very small amounts, were found in wheat flour suspensions (3).

The enzymatic oxidation of linoleic acid by lipoxygenase and the different pathways of the conversion of the primarily-formed hydroperoxides into the secondary oxidation products in suspensions of cereal flours can be represented by the following:



In contrast to previous studies made with linoleic acid added to aqueous wheat flour suspensions, the oxidation of fatty acids naturally present in flours of various cereals was studied. This was done to elucidate the contradictory results obtained by Morrison (9) and Graveland (1).

Morrison reported that, during mixing in wheat flour suspensions and doughs, both the saturated and unsaturated free fatty acids are oxidized. According to his views, the saturated fatty acids are broken down completely by a β-oxidation, while the polyunsaturated fatty acids are oxidized both by a β-oxidation and by the enzyme lipoxygenase. In contrast to Morrison, previous studies showed only the oxidation of polyunsaturated acids by the action of the enzyme lipoxygenase. The studies, however, were made with added linoleic acid, and the fate of the saturated fatty acids was not checked quantitatively. Therefore, a complementary study was made of the oxidations of

the free fatty acids naturally present in wheat flour. Hopefully, more information would be obtained about the types of oxidation products which are formed from linoleic acid in suspensions of various cereals and about the oxidation of sulfhydryl (SH) groups of the proteins by the lipoxygenase/polyunsaturated fatty acids system.

The variety of products, defined above, formed by the hydroperoxide-decomposing enzymes caused some difficulty in the evaluation of a method for lipoxygenase activity determination. An improved extraction method was developed for quantitative isolation of the free fatty acids and their oxidation products from aqueous cereal suspensions and wheat flour doughs. A gas chromatographic method for the detection of different components also was developed. After extraction, the carboxy groups of the products are methylated (10) and the hydroxy groups silylated (11), then determined by gas liquid chromatography (GLC) with the aid of an internal standard.

MATERIALS AND METHODS

Flours and Substrate

The cereals investigated were wheat, barley, rye, oats, and maize, all grown in the Netherlands. Flour samples were prepared in a Brabender laboratory mill and the flour sieved over a 0.55 mm sieve. The whole wheat meal was obtained by grinding the wheat, as was used for making flour. The substrate was linoleic acid supplied by Fluka, Switzerland.

Enzymatic Oxidation

To study the enzymatic oxidation reactions in cereal flour suspensions, 1 g of flour was suspended in 10 ml of water and incubated at 30 C under continuous magnetic stirring.

Extraction of Oxidation Products

The oxidation products in a flour-water or flour-buffer suspension were extracted in the following way. To the reaction mixture (1 g flour in 10 ml water or buffer), 4 g urea was added. The mixture was shaken thoroughly with a polytron (20 ST-OD) homogenizer (Kinematica GMBH, Luzern, Switzerland) for 15 sec. Subsequently 60 ml of a chloroform-isopropanol mixture (4:1) was added and the total mixture homogenized with the polytron homogenizer during 15 sec. Heptadecanoic acid (1 mg) dissolved in chloroform was added as an internal standard. After centrifugation, the mixture settled into 2 phases. The upper small water-isopropanol phase containing flour residues was removed, and the remaining chloroform-isopropanol phase containing lipids, small amounts of urea, and oxidation products was dried over anhydrous Na_2SO_4 and filtered. The clear extract was concentrated to 10 ml with a rotary evaporator.

Methylation of the Oxidation Products

To the extracts (ca. 10 ml), 2 ml of diazomethane-ether solution was added. After 5 min the solvent was evaporated completely with a rotary evaporator. A white precipitate containing urea, lipids, and oxidation products stayed. For the extraction of the methylated oxidation products from the white precipitate, 10 ml of 0.4N HCl was added by which the urea was dissolved. Next the oxidation products and lipids were extracted with 30 ml of chloroform. The chloroform was evaporated and the residue dissolved in 1 ml of pyridine.

Preparation of Trimethylsilyl Ether Derivatives

To enable gas chromatographic determination of mono, di, and trihydroxy acids, it was necessary to silylate all free hydroxy groups.

To the methylated oxidation products dissolved in 1 ml pyridine, 0.6 ml hexamethyldisilazane and 0.3 ml trimethylchlorosilane were added. After 15 min the reagents were removed in a stream of N_2 and the residue dissolved in 0.1 ml hexane. Insoluble material was removed by centrifugation. The sample then was purified on a Silica Gel (Camag DO) column of 2 cm height and 0.5 cm diameter using diethylether (5 ml) as an eluent. The clear extract was injected into the gas chromatograph.

Gas Chromatography

A Fractovap model GV gas chromatograph (Carlo Erba Co.) with dual flame ionization detectors was employed. Chromatography was carried out using 1 m stainless steel columns of 0.2 cm internal diameter. The packing used was 3% JXR (a silicone from Applied Science Laboratories, Inc. catalog 16, 1973) on Gas chrom Q 100/120 mesh. Temperature: 170-230 C, Prog: 1.5 C/min. Flow rate: 35 ml/min.

The quantities of the various components in the gas chromatogram were determined on the basis of the internal standard by multiplying the area of each peak by a response factor, depending upon the number of hydroxyl groups in each component. The response factors are determined with the aid of pure substances. The response factors are: trihydroxy acids = 0.85, dihydroxy acids and ketodihydroxy acids = 0.90, monohydroxy acids and ketols = 0.95.

Determination of Hydroperoxides

The gas chromatographic method for the

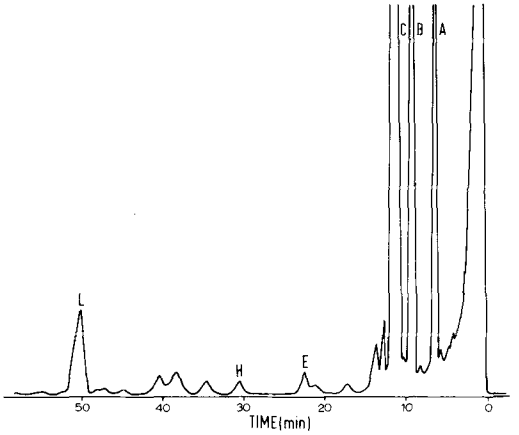


FIG. 1. Gas chromatogram of the free fatty acids extracted from normal wheat flour and their oxidation products.

determination of oxidation products can be applied when the extract does not contain hydroperoxides, which do not, like silylated products, stand the high temperature of the gas chromatograph. Therefore, the extracts must be checked beforehand by TLC on the absence of hydroperoxides.

The methylated extracts were applied on a TLC plate (the plates are made the same way as described in a previous publication [2]) and chromatographed as follows: the plate was first developed up to 6 cm in a mixture of diethyl-ether-benzene-ethanol-acetic acid (40:50:2:0.2). After drying, the plate was further developed in the same direction in a mixture of isooctane-ether (50:50). The spots are charred by spraying the plates with 25% H_2SO_4 and subsequent heating for 10 min at 200 C. The band just preceding the free fatty acids consists of hydroperoxides.

Reduction of the Keto Group

The keto group present in α-hydroxy-keto acids, γ-hydroxy-keto acids, and ketodihydroxy acids is reduced to the corresponding hydroxy group by the following treatment: 2 mg of the methylated component dissolved in 4 ml of a chloroform-ethanol mixture (6:1) and 10 mg $NaBH_4$ added. The reaction mixture was shaken 30 min at room temperature. Then, 3 ml chloroform was added, the mixture centrifugated, and 1 ml water added. The total mixture was shaken carefully. After the mixture had settled into 2 phases, the upper water-ethanol phase was removed, and the remaining chloroform-ethanol phase, containing the reduced products, was dried over anhydrous Na_2SO_4.

RESULTS

Figure 1 shows the gas chromatogram of the chloroform-methanol extract of normal wheat flour. This chromatogram contains 3 large peaks from the free fatty acids as methyl esters. The first peak (A) is palmitic acid, the second peak (B) the internal standard heptadecanoic acid, and the third peak represents a mixture of stearic-, oleic-, linoleic-, and linolenic acids. a JXR column separates only the fatty acids with different chain lengths. The quantities of the various free fatty acids from flour are determined as methyl esters with a 2 m glass column, packed with 10% Sp-222-ps on 100/120 mesh support (Supelco, Pa.). The results are shown in Table I. The other small peaks in the gas chromatogram are explained in the discussion of Figure 2.

TABLE I

The Quantities of the Free Fatty Acids (mg/100 g flour)

Palmitic acid	54 mg
Stearic acid	4 mg
Oleic acid	35 mg
Linoleic acid	146 mg
Linolenic acid	8 mg
Total	247 mg

Figure 2 shows the gas chromatogram of the extract from an aqueous wheat flour suspension which was shaken during 15 min. This picture is much different from Figure 1. In this scan, peak C has decreased, while a number of new peaks have emerged. These new peaks are oxidation products of linoleic and linolenic acid oxidation during shaking of the suspension. Because linolenic acid is present in a small quantity in relation to linoleic acid, the contribution of the oxidation products from linolenic acid is correspondingly small. The oxidation

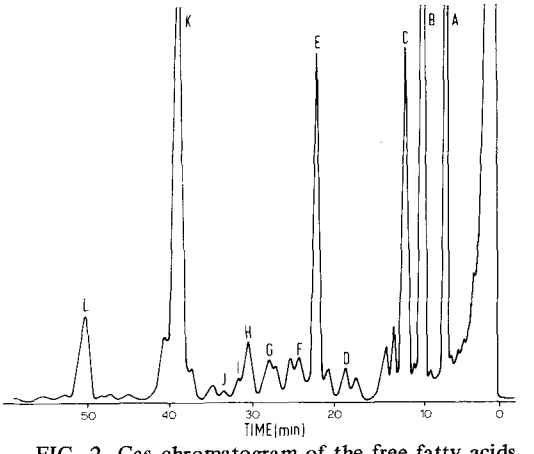


FIG. 2. Gas chromatogram of the free fatty acids extracted from a suspension of normal wheat flour in water and their oxidation products.

TABLE II

Oxidation Products of Linoleic Acid Extracted from Suspensions of Various Cereals

Oxidation products	Peak GLC	Normal wheat flour	Suspension of normal wheat flour	Suspensions of cereals, 2 mg linoleic acid added to 1 g of defatted flour				
				Wheat	Barley	Rye	Oats	Maize
Linoleic acid (remaining after reaction)	C	146[a]	30	120	100	130	120	140
Unknown	D		2	3	1	1	2	1
Monohydroxy acids	E	2	25	25	15	15	50	10
Keto acids	F		3	0.5		0.5	0.1	
Ketohydroxy acids	G		3	0.2	50	30		40
Dihydroxy acids	H	1	5	2	7	7	5	2
Dihydroxy acids	I		0.5	0.1			1	
Ketodihydroxy acids	J				2		1	
Trihydroxy acids	K		75	46	20	10	20	1

[a]Figures = mg of the oxidation product/100 g flour.

products of linolenic acid have the same retention times as the oxidation products of linoleic acid.

With the aid of pure substances, which were isolated with preparative TLC and identified (2), the peaks in the gas chromatogram have been verified. Peak E represents monohydroxy acids, the 9-hydroxy-10*trans*, 12*cis*-octadecadienoic acid (85%), and 13-hydroxy-9*cis*, 11*trans*-octadecadienoic acid (15%). These quantities have been estimated with TLC (2). Peak F is a keto acid; after reduction with $NaBH_4$ the peak shifts to the place of monohydroxy acids, so it appears to be a keto acid having 18 C atoms, probably 9-oxo-octadeca-9,11-dienoic acid (12). The exact structures have not been elucidated yet.

Peak G comprises unresolved α and γ-ketols. Besides, both types of ketols contain 2 isomers. The α-ketols are 9-hydroxy-10-oxo-12*cis*-octadecenoic acid (90%) and 12-oxo-13-hydroxy-9*cis*-octadecenoic acid (10%); the γ-ketols are 10-oxo-13-hydroxy-11*trans*-octadecenoic acid (90%) and 9-hydroxy-12-oxo-10*trans*-octadecenoic acid.

Peak H consists of 2 isomers of dihydroxy acids, of which the 2 hydroxy groups are vicinal: 9,10-dihydroxy-12*cis*-octadecenoic acid (85%) and 12,13-dihydroxy-9*cis*-octadecenoic acid (15%).

Peak I consists of 2 isomers of dihydroxy acids, whose 2 hydroxy groups are not vicinal: 9,12-dihydroxy-10*trans*-octadecenoic acid and 10,13-dihydroxy-11*trans*-octadecenoic acid.

Peak J consists of 2 ketodihydroxy acids: 9,10-dihydroxy-13-oxo-11*trans*-octadecenoic acid and 9-oxo-12,13-dihydroxy-10*trans*-octadecenoic acid. Peak K consists of trihydroxy acids: 9,12,13-trihydroxy-10*trans*-octadecenoic acid (90%) and 9,10,13-trihydroxy-11*trans*-octadecenoic acid (10%). The small peaks with retention times of 35, 37, and 41 are not oxidation products. Peak L consists of monoglycerides, which contain stearic, oleic, linoleic, and linolenic acids.

Whether peak G represents α or γ-ketols can be established by reducing the total extract with $NaBH_4$ after methylating and silylating. The α-ketols then will be converted into the dihydroxy acids of the type represented by peak II, the γ-ketols into those of peak I. Between the peaks F and G are 2 small unknown peaks. Reduction with $NaBH_4$ does not affect the retention times. The same is true for the unknown peak D.

In Table II, column 4, the quantities of various oxidation products are given. It is interesting to observe that the sum of the oxidation products is equal to the decrease of the amounts of linoleic and linolenic acids. These results provide evidence, first that the polyunsaturated fatty acids are oxidized only by lipoxygenase and second that the saturated fatty acids are not oxidized.

To obtain more information about the enzymatic oxidation of polyunsaturated fatty acids and to check this method, the enzymatic oxidation of linoleic acid in other cereals, barley, oats, rye, and maize, were studied. The free fatty acids content varies considerably in these cereals (1-4 mg per g of flour). For that reason, the flours are extracted by petroleum ether; then 2.0 mg of linoleic acid is added to a suspension of 1 g of the extracted flour in 10 ml water.

Figure 3 shows the gas chromatograms of the oxidation products found in the different suspensions. The gas chromatograms from

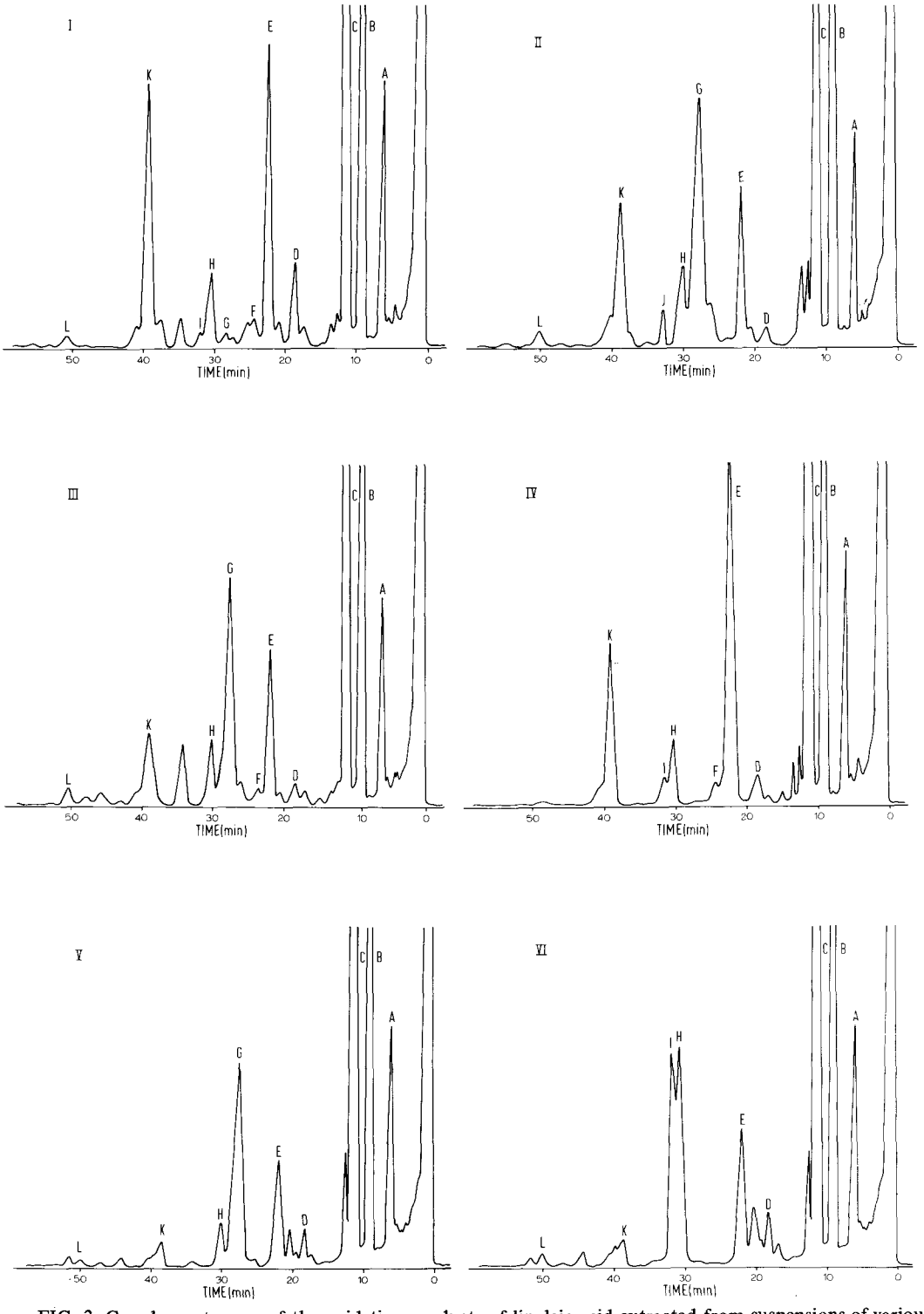


FIG. 3. Gas chromatogram of the oxidation products of linoleic acid extracted from suspensions of various cereals. I = wheat flour, II = barley flour, III = rye flour, IV = oat flour, V = maize flour, VI = extract of V after reduction with $NaBH_4$.

wheat (I), barley (II), and rye (III) are similar. This similarity is also evident in Table II. Striking features are the high percentages of trihydroxy acids (peak K) in wheat and those of ketols (Peak G) in barley and rye. The gas chromatograms of oats (IV) and maize (V) are simpler. In a suspension of oats, great quantities of monohydroxy acids have been formed, while in a maize suspension great quantities of ketols have been formed. Gas chromatogram VI is the extract from maize after reduction with $NaBH_4$; peak G was found to consist of equal amounts of α and γ-ketols.

With the aid of TLC it was found that in all cases peak E consists of ca. 90% of 9-monohydroxy acid and ca. 10% of 13-monohydroxy acid. From these results it may be concluded that the other secondary oxidation products which are formed in suspensions of rye, oats, and maize consist of 2 isomers and probably in the same ratio as they occur in a wheat suspension.

Table II and Figure 3, show that the lipoxygenase activity is different for various cereals under the conditions of the present study and that the subsequent reactions, which are responsible for the conversion of hydroperoxides or peroxy radicals into secondary oxidation products, can differ widely. That means that the isomerase activities, the lipoxygenase activity, and the glutenin-induced catalytic activity can vary widely. In an oatmeal suspension no ketols are formed, but great quantities of monohydroxy acids were, whereas in a rye flour suspension ketols, and less monohydroxy acids are formed. In a whole wheat meal suspension, more dihydroxy acids and ketohydroxy acids are formed than in a suspension of wheat flour. The formation of the products in a suspension of whole wheat meal points out that, in the outer layers of the wheat kernel (which are removed when the wheat is milled into flour), isomerases occur which are responsible for the formation of dihydroxy acids and ketohydroxy acids. Whether these components are formed by different isomerases is still a question. It could be established that the dihydroxy acids are not formed from ketohydroxy acids but directly from hydroperoxides.

In a suspension of wheat flour 0.9, μmol monohydroxy acids are formed, so 0.9 μmol hydroperoxides are reduced. When SH groups (the wheat flour used for this experiment contained 1.0 μmol SH groups/g flour) are blocked with N-ethylmaleimide, nearly the same quantity of monohydroxy acids are formed. In other words, the reduction of hydroperoxides probably is not affected by the SH groups of protein only but also by the enzyme lipoperoxidase (8).

DISCUSSION

In experiments in aqueous cereal suspensions, it was found that the sum of the oxidation products is equal to the decrease in the amount of linoleic and linolenic acids, whereas the quantity of the saturated acids did not change. From these facts it may be inferred that only the polyunsaturated fatty acids are oxidized. The decrease of saturated fatty acids, reported by Morrison (9), may well be attributed to an incomplete extraction. Upon contact of wheat flour with water the flour proteins tend to lock in all the free fatty acids and their oxidation products, because the latter have a hydrophobic alkyl chain and a free carboxyl group. As a result they become difficult to extract. A number of preliminary experiments, in which pure wheat protein fractions were mixed with fatty acids, proved that a sturdy complex is formed including both proteins and fatty acids. This complex can be disclosed by adding urea to the suspension. For a complete disclosion of the free fatty acids included in the complex, the concentration of urea must be at least 6M.

The manner in which the fatty acids are linked to the proteins is a question. It is possible that the alkyl chains of the free fatty acids interact, by their hydrophobic character, with the nonpolar side chains of the proteins. This may result in the formation of water soluble or insoluble mixed micellar systems of fatty acid and protein molecules.

The gas chromatographic method described offers a means of determining all the nonvolatile oxidation products; this method is sensitive. It has the drawback, however, that it does not permit the determination of hydroperoxides and volatile oxidation products. Compared with other methods (titrimetric [13], polarographic [14], or spectrophotometric [15] ones) the present gas chromatographic method does furnish the most information about both the lipoxygenase activity and the activities of the hydroperoxide-decomposing enzymes in a natural system.

St. Angelo et al. (16) developed a gas chromatographic method for determining volatile lipoxygenase reaction products. Combining this method for volatile products and the present method for nonvolatile products may furnish useful information about the mechanism of the formation of volatile products, which have been found in bread and other foods.

The present study comprises only aqueous suspensions, having pH 6.5. It is quite possible that at different pH, the activities of the

enzymes, participating in the sequence of reactions shown in the introduction, may change in relation to each other. This will result in a change of the proportions of the oxidation products.

The conversion of hydroperoxides to monohydroxy isomers is not elucidated yet. Nearly the same quantity of monohydroxy isomers is formed in a suspension of wheat flour after blocking the SH groups with N-ethylmaleimide and without blocking. The question, however, is to what extent SH groups have been blocked by the addition of N-ethylmaleimide. If glutathion is added, more monohydroxy isomers are formed and less of the other secondary oxidation products. This indicates a reduction of hydroperoxides to monohydroxy isomers. Gardner (17) also proved a reduction of hydroperoxides by cysteine with ferric ions as a catalyst. Heiman et al. (17) suggests an enzymatic reduction in an oat flour suspension of hydroperoxides to monohydroxy isomers by the enzyme lipoperoxydase. Further study is needed to elucidate the exact mechanism of the conversion of hydroperoxides into monohydroxy isomers.

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REFERENCES

1. Graveland, A., JAOCS 47:352 (1970).
2. Graveland, A., Technical Quarterly 9:98 (1972).
3. Graveland, A., Paper presented at the 11th ISF Congress, Göteborg, Sweden, (1972).
4. Zimmerman, D.C., Biochem. Biophys. Res. Commun. 23:398 (1966).
5. Veldink, G.A., J.F.G. Vliegenthart and J. Boldingh, Biochem. J. 120:55 (1970).
6. Gardner, H.W., J. Lipid Res. 11:311 (1970).
7. Chang, C.C., W.J. Esselman and C.O. Clagett, Lipids 6:100 (1971).
8. Heimann, W., F. Reiartz and P. Schreier, Helv. Chim. Acta. 55:2257 (1972).
9. Morrison, W.R., and E.A. Maneely, J. Sci. Food Agr. 20:379 (1969).
10. Schlenk, H., and J.L. Gellerman, Anal. Chem. 32:1412 (1960).
11. Wood, R.D., P.K. Raju and E. Reiser, JAOCS 42:161 (1965).
12. Garssen, G.J., J.F.G. Vliegenthart and J. Boldingh, Biochem. J. 122:327 (1971).
13. Hadron, H., K.W. Biefer and H. Suter, Z. Lebensm. Unters. Forsch. 104:316 (1956).
14. Mitsuda, H., K. Yasumoto and A. Yamamoto, Agr. Biol. Chem. 31:115 (1967).
15. Thaler, H., H.J. Kleinau, Die Nahrung. 11:685 (1967).
16. St. Angelo, A.J., H.P. Dupuy and R.L. Ory, Lipids 7:93 (1972).
17. Gardner, H.W., R. Kleinman and G.E. Inglett, JAOCS 50:85A (1973).

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Enzymatic Oxidation of Linolenic Acid in Aqueous Wheat Flour Suspensions

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ABSTRACT

Linolenic acid oxidation by the enzyme lipoxygenase in an aqueous wheat flour suspension does not lead to accumulation of linolenic acid hydroperoxides but immediately to secondary oxidation products. The 3 most important products among these were identified as 9-hydroxy-*trans*-10,*cis*-12,*cis*-15-octadecatrienoic acid, 9-hydroxy-10-oxo,*cis*-12,*cis*-15-octadecadienoic acid, and 9,12,13-trihydroxy-*trans*-10,*cis*-15-octadecadienoic acid.

INTRODUCTION

Lipoxygenase has been shown to attack a number of polyunsaturated fatty acids. The enzyme attack is highly specific: it reacts with acids which have a *cis,cis*-1,4-pentadiene system, such as linoleic, linolenic, and arachidonic acid, to produce hydroperoxides with a *cis,trans* conjugated diene system (1).

In a previous publication (2) it was reported that lipoxygenase from wheat flour forms mainly the 9-hydroperoxide (or maybe 9-peroxy-radical) from linoleic acid. This 9-hydroperoxide (or 9-peroxy-radical) is converted immediately into various secondary oxidation products (3).

The free fatty acids of wheat flour include up to 4-5% linolenic acid, i.e. ca. 0.05 mg/g flour. This paper describes the identification of 3 oxidation products from linolenic acid by IR, NMR, and mass spectrometry.

MATERIALS AND METHODS

Flour and Substrate

A commercial wheat flour was used, which contained no bleaching nor improving agent and which had a protein content (N x 5.7) of 10.1% (on 14.0% moisture basis). Linolenic acid was obtained from Fluka, Switzerland.

Enzymatic Oxidation of Linolenic Acid

To study the oxidation of linolenic acid in wheat flour suspensions, 1 g wheat flour defatted with petroleum ether was suspended in 10 ml water, 2 mg linolenic acid added, and the mixture incubated under continuous magnetic stirring at 30 C. After methylation and silylation, the products were determined by gas chromatography (3). The linolenic acid remaining in the flour after extraction, amounting to some 0.01 mg/g flour, is not considered.

For preparative isolation of the oxidation products, a suspension of 20 g defatted flour and 40 mg linolenic acid in 100 ml was incubated.

Extraction and Isolation of Oxidation Products

After incubation of the reaction mixture, the oxidation products were extracted with a chloroform-isopropanol mixture (3). For preparative isolation of the oxidation products, preparative thin layer chromatography (TLC) was used. After applying the extract on the plate with a Camag applicator, the plate was first developed in a solvent mixture of diethylether-benzene-ethanol-acetic acid (40:50:2:0.2) up to 6 cm. After drying for 5 min, the plate was developed in the same direction (15 cm) in the following solvent mixture: isooctane-diethylether-acetic acid (50:50:2). After chromatography the components were made visible on the TLC plate by means of iodine vapor, scraped off, and transferred into a column. The components were eluted from the silica gel with ether.

IR, NMR, and Mass Spectra Analysis

IR spectra were obtained with a Hitachi EPG-3. The analyses were carried out in either solution of CCl_4 or KBr pellet form. NMR spectra were obtained with a JNM-4H-60 spectrometer at 25 and 45 C. The analyses were carried out in $CDCl_3$ solution. Mass spectra were obtained with an Atlas mass spectrometer, type CH-4, equipped with a TO 4 ion chamber. Mass measurements were carried out with an Atlas double focusing mass spectrometer SM-1.

Methylation and Silylation of the Oxidation Products

For analysis, the isolated oxidation products were methylated with diazomethane and silylated with hexamethyldisilazane and trimethylchlorosilane (3).

Gas Chromatography

The methylated and silylated products were determined by gas chromatography. Chroma-

tography was carried out using 1 m stainless steel columns of 0.2 cm internal diameter. The packing used was 3% JXR on Gas Chrom Q 100-120 mesh. Temperature: 170-230 C, Prog. 1.5 C/min.

RESULTS

Figure 1 shows the gas chromatogram of methyl esters derived from an extract of a suspension containing linolenic acid and defatted wheat flour after 30 min shaking. The first peak A is the methyl ester of palmitic acid, which is not extracted by petroleum ether. The second peak B is the methyl ester of the internal standard (heptadecanoic acid), while the third peak C represents a mixture of methyl stearate, oleate, linoleate, and linolenate, which are not extracted by petroleum ether, plus the methyl ester of linolenic acid which was added to the suspension and not oxidized. Further, there are 3 large peaks to be attributed to oxidation products, E, G, and K. These products have been isolated by preparative TLC and identified.

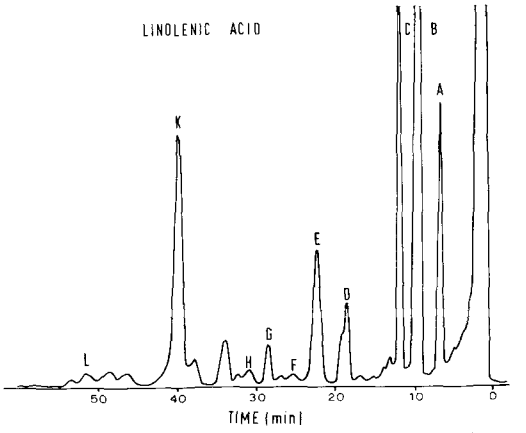


FIG. 1. Gas chromatogram of oxidation products from linolenic acid. A = palmitic acid, B = heptadecanoic acid (standard), C = linolenic acid, D = unknown, E = monohydroxy isomer of linolenic acid, F = unknown, G = α-ketol isomer of linoleic acid, H = unknown, and K = trihydroxy isomer of linolenic acid. These products were all methylated and oxidation products methylated and silylated.

9-Hydroxy-*trans*-10,*cis*-12,*cis*-15-octadecatrienoic Acid (E)

The IR spectrum (Fig. 2A) of the methylated product indicates the presence of a methyl ester (1735 and 1170 cm^{-1}), a *cis,trans* conjugated double bond (3010, 982, and 950 cm^{-1}), and a hydroxy group (3600 cm^{-1} sharp peak and a broad band with a top at 3450 cm^{-1}). The small peak at 1070 cm^{-1} indicates a secondary hydroxy group.

The mass spectrum (Fig. 3) of this methylated and silylated compound shows a small parent (p) peak (m/e 380). Furthermore, the spectrum contains the peaks with m/e values of p15 and p31, attributable to a loss of CH_3 and CH_3 + CH_4 of the trimethylsilyl (TMS) group (4). The p31 also may be attributed to a loss of

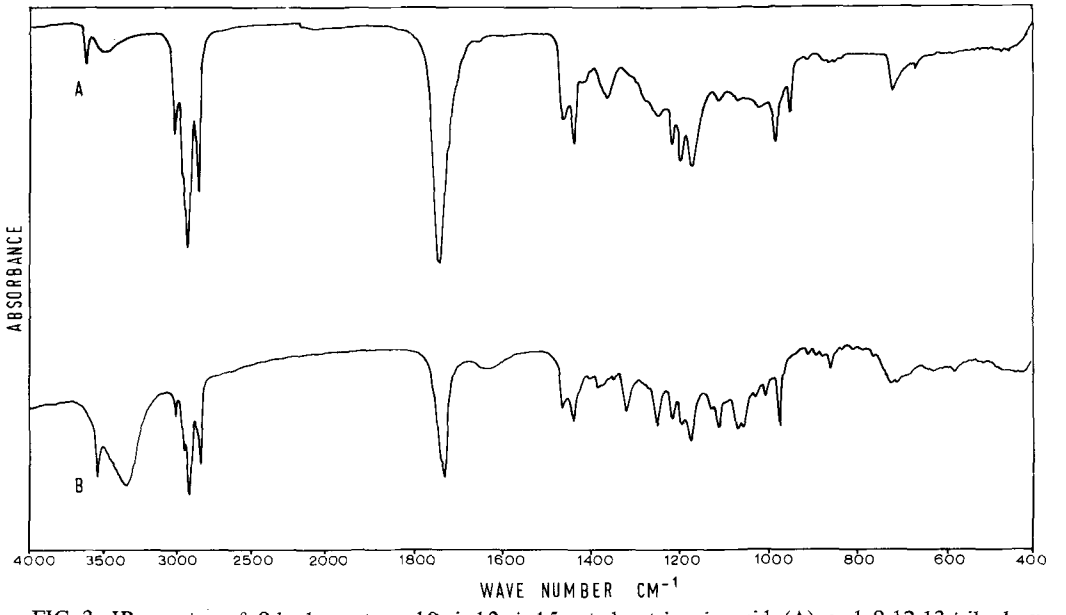


FIG. 2. IR spectra of 9-hydroxy-*trans*-10,*cis*-12,*cis*-15-octadecatrienoic acid (A) and 9,12,13-trihydroxy-*trans*-10,*cis*-15-octadecadienoic acid (B). A in CCl_4 using 0.1 mm KBr cell; B in KBr pellet form.

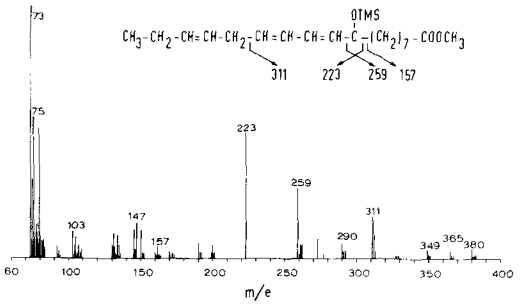


FIG. 3. Mass spectrum of 9-hydroxy-*trans*-10,*cis*-12,*cis*-15-octadecatrienoic acid after methylation and silylation.

the OCH_3 group. Peaks 157, 223, and 259 correspond to the fragments indicated in the formula in Figure 3. These peaks show the location of the TMS group on carbon-9. Peak 311 defines the location of the conjugated dienol system and locates the double bonds in the 10 and 12 positions (5). In this spectrum, there are no peaks which indicate an isomer of this compound. Also with TLC of the methyl hydroxy stearate derivative, it was shown that only the 9-hydroxy isomer was present.

Further, this mass spectrum, like the other mass spectra in this paper, contains a number of peaks which are characteristic of silylated compounds, 73 ($Si[CH_3]_3^+$); 75 ($HO^+ = Si[CH_3]_2$); 103 and 147.

9-Hydroxy-10-oxo,*cis*-12,*cis*-15-octadecadienoic Acid (G)

After reduction with $NaBH_4$ peak G shifts to the place of peak H. This change of retention time indicates the presence of a keto group (3).

The IR spectrum provides no indication of a *cis,trans* conjugation, as no peaks are found at 950 cm^{-1} or at 982 cm^{-1}. Also there is no indication of an isolated *trans* double bond at 970 cm^{-1}. The IR spectrum of the methylated compound indicates the presence of a methyl ester (1735 and 1170 cm^{-1}), a ketone group (1720 cm^{-1}), a hydroxyl group (3490 cm^{-1} and 1075 cm^{-1}), and *cis* double bonds (3010 cm^{-1}). From these results and from the location of this compound in the gas chromatogram, it may be inferred that this compound is a keto-hydroxy acid.

The tentatively established structure of this keto-hydroxy acid was confirmed by the mass spectrum (Fig. 4). It shows a p peak (m/e 396) and 381 (p15), 365 (p31), and 349 (p47). The 2 peaks 239 and 259 correspond to the fragments shown in the formula in Figure 4. Further, there is a large peak 169 (259-TMS). The peaks 259 and 169 establish the location of the TMS group on C-9.

After reduction with $NaBH_4$ followed by oxidative chain cleavage with $NaJO_4$ (2), a reaction product was obtained which was identified by gas chromatography as $OCH(CH_2)_7COOCH_3$. This leads to the conclusion that the keto group is located on C-10. There are no other peaks in the mass spectrum which may indicate any isomer. So it may be inferred that peak G in the gas chromatogram (Fig. 1) is to be attributed only to 9-hydroxy-10-oxo,*cis*-12,*cis*-15-octadecadienoic acid.

9,12,13-Trihydroxy-*trans*-10,*cis*-15-octadecadienoic Acid (K)

The IR spectrum (Fig. 2B) of the methylated product indicates the presence of a methyl ester (1730 and 1173 cm^{-1}), a *cis* double bond (3000 cm^{-1}), and an isolated *trans* double bond (970 cm^{-1}). Hydroxy groups are indicated by a sharp peak at 3520 cm^{-1} and a broad band with a top at 3350 cm^{-1}. The peaks

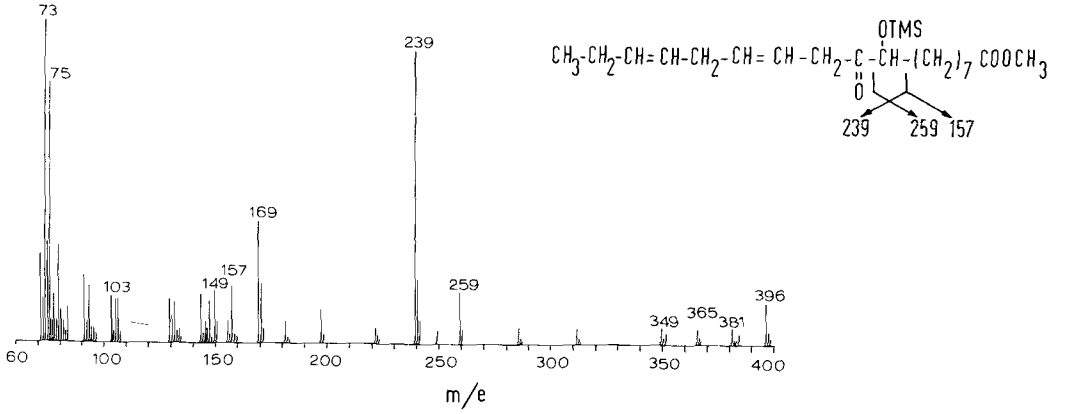


FIG. 4. Mass spectrum of 9-hydroxy-10-oxo,*cis*-12,*cis*-15-octadecadienoic acid after methylation and silylation.

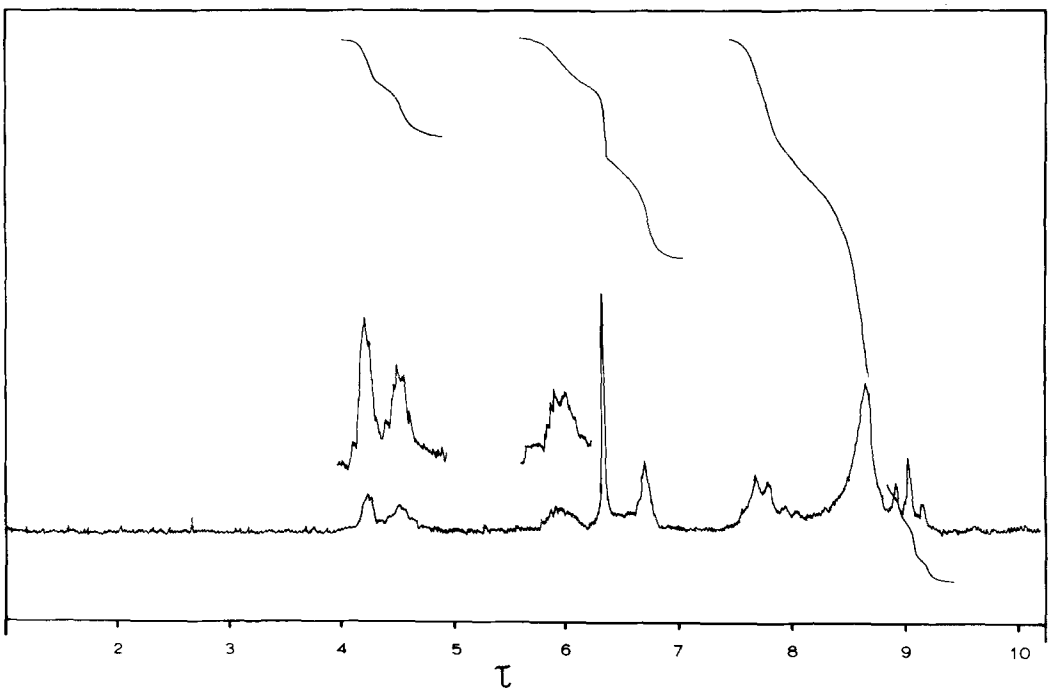


FIG. 5. NMR spectrum of 9,12,13-trihydroxy-*trans*-10,*cis*-15-octadecadienoic acid after methylation.

at 1050 and 1070 cm^{-1} indicate more than 1 secondary hydroxy group.

With the aid of the NMR spectrum (Fig. 5) the number of hydroxy groups could be detected. The NMR spectrum of the methylated compound is made up as follows: a triplet at 9.1 τ (3H; terminal methyl), a broad peak with top at 8.7 τ (12H, $[CH_2]_6$-), a multiplet between 7.6 and 8.6 τ (6H CH_2-$COOCH_3$ and 2 x CH_2-C-OH), a broad peak with top at 6.7 τ (3H HO-C), a broad peak between 6.4-6.8 τ (1H H-COH), a singlet at 6.3 τ (3H OCH_3), a broad peak between 5.8 and 6.3 τ (2H H-COH), a multiplet with top at 4.5 τ (2H HC=CH), and a multiplet at 4.2 τ (2H HC=CH).

This spectrum was photographed in $CDCl_3$ at 25 C. When the spectrum was photographed at 45 C, the OH peak shifted from 6.7 τ to 7.4 τ; and, when the sample was shaken with D_2O, the peak disappeared. From these results it may be inferred that there are 3 hydroxy groups and 2 double bonds. From comparison with the NMR spectrum of 9,12,13-trihydroxy-10(*trans*)-octadecenoic acid (6), it may be concluded that there is 1 *trans* double bond 4.2 τ and 1 *cis* double bond 4.5 τ.

The locations of the 3 hydroxy groups were determined by mass spectrometry. In the mass spectrum (Fig. 6), the p peak 558 is small. The characteristic TMS peaks 73, 75, 103, and 147 are present. Further striking features are 543 (p15) and 527 (p31). The peaks 259, 387, and 171 correspond with the fragments shown in Figure 6. Peak 399 ($C_{20}H_{39}O_4Si_2$) is 489-90. Peak 489 (not occurring in the spectrum) is p-(CH_3-CH_2-CH=CH-CH_2-). The structure of the substance represented by peak 460 is shown in Figure 6 (5).

It has been assumed that the *trans* double bond in the components E and K is *trans*-10, though this has not been proved exactly. By comparison, however, with the products obtained from linoleic acid using the same enzyme system (2), it may be assumed that in the above named products the *trans* double bond is located on C-10.

DISCUSSION

In an aqueous wheat flour suspension linolenic acid is enzymatically oxidized, which results in the formation of 3 major compounds E, G, and K; these have been identified. Smaller quantities of D, F, and H also are formed; the structure of these compounds has not been identified.

From the structures of the compounds E, G, and K, it appears that the positional specificity in the oxygenation of polyunsaturated fatty acids by lipoxygenase from wheat flour increases when the acid is more unsaturated. Lipoxygenase oxidizes linoleic acid primarily at

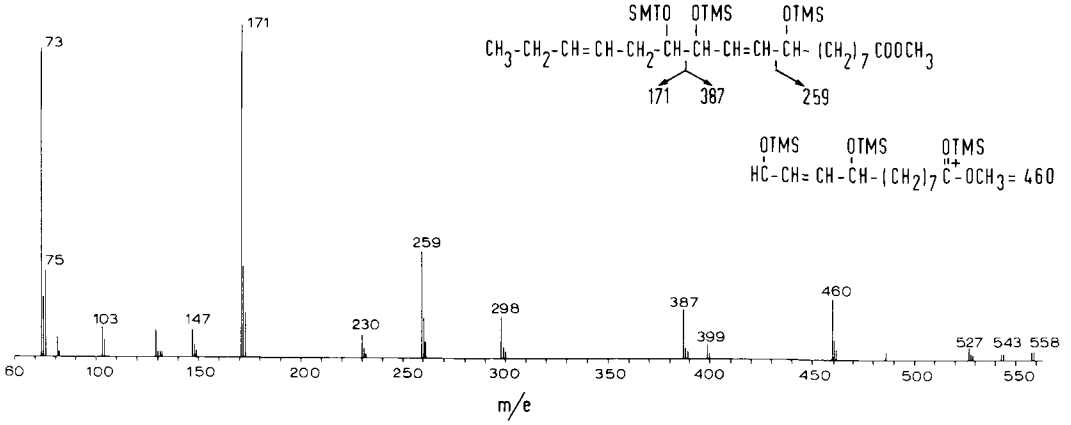
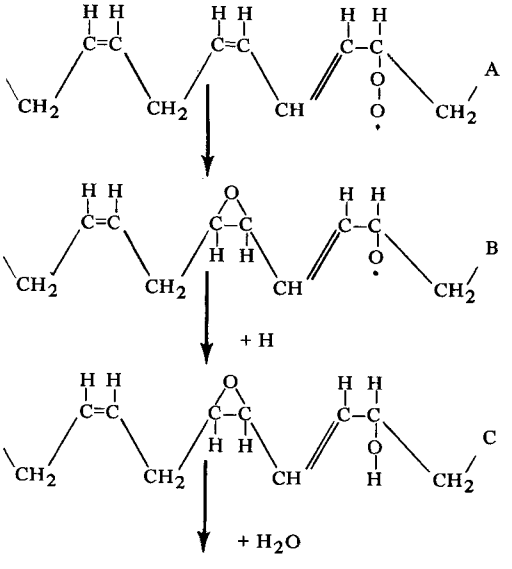


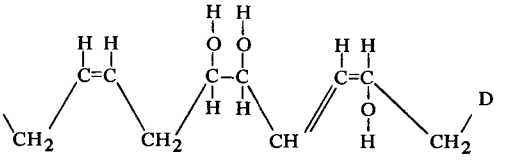
FIG. 6. Mass spectrum of 9,12,13-trihydroxy-*trans*-10,*cis*-15-octadecadienoic acid after methylation and silylation.

carbon-9 (85%) with a small amount (15%) of oxidation at carbon-13 (2), whereas in linolenic acid only oxidation at carbon-9 occurs, while no oxidation takes place at carbon-16, -12, and -13.

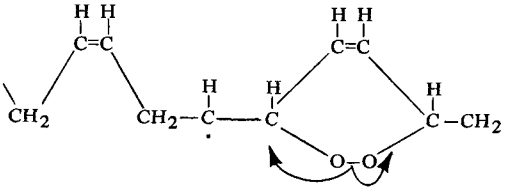
The location of the 3 hydroxy groups in the molecule of trihydroxy acid (K), carbons-9, -12, and -13 is the same as in the quantitatively predominant trihydroxy acid isomer of linoleic acid. From this it may be inferred that the formation of this trihydroxy acid takes place in the same way as it does in linoleic acid, via a hydroxy-epoxy compound. In previous experiments (6), it was found that the hydroxy-epoxy acids are formed from the hydroperoxy acids or peroxy radicals.

The conversion of the peroxy radical of linolenic acid hydroperoxide into a trihydroxy acid may be represented by the scheme below:





The peroxy radical (A) probably is transformed by rearrangement into an epoxy-alkoxy radical (B). The exact mechanism of this rearrangement is still obscure. In the event of a direct formation of an epoxy-alkoxy radical from a peroxy radical via a radical mechanism, a six membered ring with 1 double bond as an intermediate would be required:



However, this double bond in the ring system necessarily has the *cis*-configuration, in contrast to what has been demonstrated, viz. that this double bond has a *trans*-configuration (6). There may be another possible pathway, the formation of a hydroxyl radical in step 1 and its subsequent addition to the double bond.

The possibility should not be excluded that the epoxy-alkoxy radical is formed indirectly; the conversion of the peroxy radical into the epoxy-alkoxy radical may occur via a number of intermediate states.

The hydroxy-epoxy acid (C) was not determined because only the oxidation in suspension was studied. In a suspension hydrolysis takes place so rapidly that it is impossible to detect it.

The formation of an α-ketol isomer (G) is indicative of the presence of the enzyme isomerase of the flax seed type, which was isolated by Zimmerman (8). In all probability, it is one and the same enzyme forming the α-ketol isomer(s) both from linoleic acid (3) and from linolenic acid in aqueous wheat flour suspensions.

The mechanism of the conversion of the linolenic acid hydroperoxide into the monohydroxy isomer (E) is not yet elucidated, as is the oxidation of linoleic acid (3).

ACKNOWLEDGMENTS

N. Visser assisted in experiments and analysis. Mass spectra were recorded by M.C. ten Noever de Braun (Central Institute for Nutrition and Food Research, Zeist) and NMR spectra by P. Smit (Agricultural University of Wageningen).

REFERENCES

1. Tappel, A.L., in "The Enzymes," Second edition, Vol. 8, Edited by P.D. Boyer, H. Lardy and K. Myrbach, Academic Press, New York, 1963, p. 275.
2. Graveland, A., Tech. Quart. 9:98 (1972).
3. Graveland, A., Lipids 8:599 (1973).
4. McCloskey, J.A., R.N. Stillwell, and A.M. Lawson, Anal. Chem. 40:233 (1968).
5. Kleiman, R., and G.F. Spencer, JAOCS 50:31 (1973).
6. Graveland, A., Ibid. 47:352 (1970).
7. Graveland, A., Ph.D. Thesis, University of Utrecht (1971).
8. Zimmerman, D.C., Biochem. Biophys. Res. Commun. 23:398 (1966).

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Detection of Aldehydes with 4-Amino-5-Hydrazino-1,2,4-Triazole-3-Thiol as Spray Reagent

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ABSTRACT

A spray reagent of 4-amino-5-hydrazino-1,2,4-triazole-3-thiol, 2% in aqueous 1N NaOH, was used for the detection of aldehydes on chromatograms of lipids. Purple spots are produced with aldehydes, and 1 μg of palmitaldehyde can be detected easily on thin layer chromatograms. The reagent is specific for aldehydes and does not react with ketones. The aldehyde moiety from plasmalogens is detected only after acidic hydrolysis. Purple or red stains arise also with ozonides. Simple precautions against autoxidation of the lipids should be taken to prevent formation of aldehydes which could lead to misinterpretations.

INTRODUCTION

Dickinson and Jacobson (1) reported formation of purple compounds from 4-amino-5-hydrazino-1,2,4-triazole-3-thiol (2) and aldehydes. They suggested the scheme of condensation and oxidation reactions shown in Table I to explain the strong coloration, and they recommended the triazole as a sensitive reagent for specific detection of aldehydes.

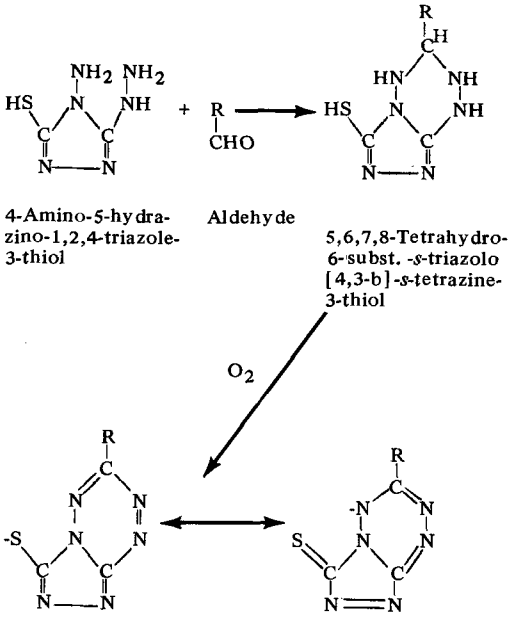
Grafe and Engelhardt applied the reagent in conjunction with periodic acid to detect nonreducing sugars on polyamide thin layer chromatograms (TLC) (3). To the authors' knowledge, further reports on the reagent have not appeared. The authors explored the application to lipid aldehydes and aldehydogenic compounds which often are only minor components of complex mixtures and, therefore, are not easily detectable.

EXPERIMENTAL PROCEDURES

Spray reagent: 4-amino-5-hydrazino-1,2,4-triazole-3-thiol (AHTT) is commercially available (Aldrich Chemical Co., Inc., Milwaukee, Wis. or Fluka AG, Buchs, Switzerland) or can be prepared from hydrazine hydrate and thiourea according to described procedures (1,2,4). A concentration of 2% AHTT in 1N NaOH was

TABLE I

Color Reaction of Aldehydes[a] (1)



[a]Nomenclature according to Chem. Abstr., 8th coll. vol. 1967-1971, Index Guide, 2030G and 2037G (1972).

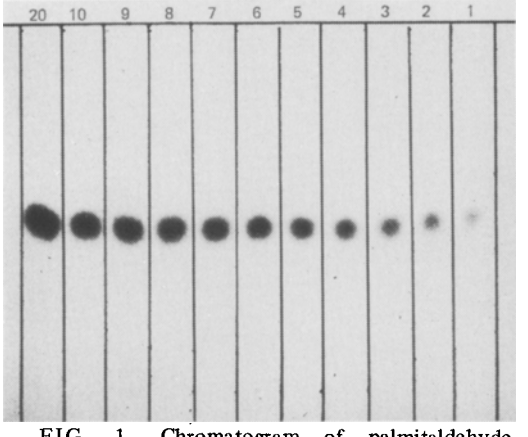


FIG. 1. Chromatogram of palmitaldehyde, amounts decreasing from 20 to 1 μg, 45 min after spraying with 4-amino-5-hydrazino-1,2,4-triazole-3-thiol.

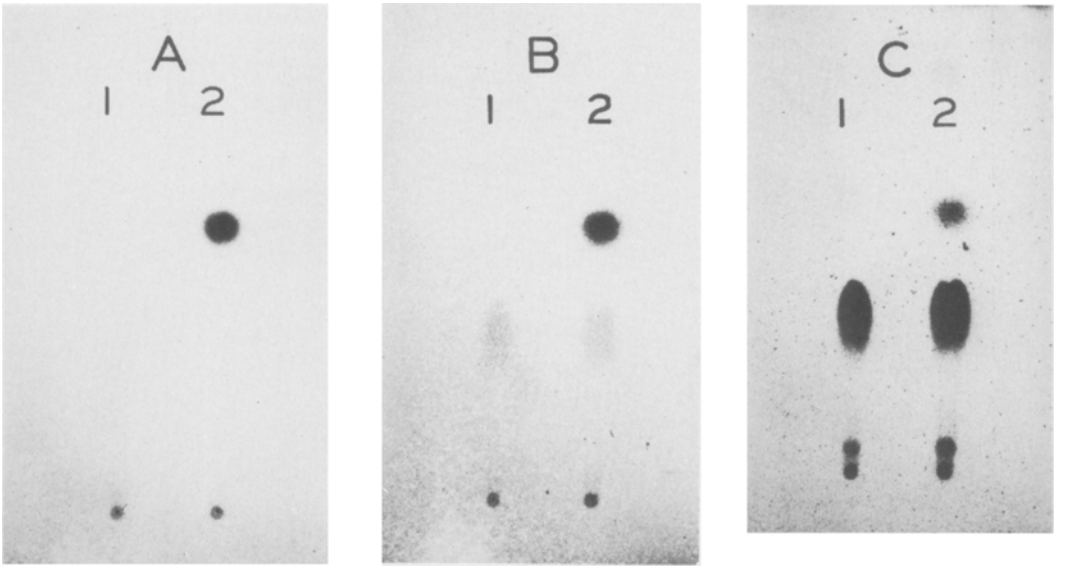


FIG. 2. Chromatogram of fish body lipids (1) without and (2) with palmitaldehyde added, 45 min after spraying with 4-amino-5-hydrazino-1,2,4-triazole-3-thiol. (A); 24 hr after spraying (B); then heat charred with $K_2Cr_2O_7/H_2SO_4$ (C).

used as spray reagent. The solution slowly turns pink, and only a small amount should be prepared at a time.

TLC plates, after development, are dried in a stream of N_2 under a plastic cover to prevent autoxidation of lipids. The plates then are sprayed with a fine mist of the reagent. The presence of aldehydes is indicated by the purple color which begins to appear within 1 min. Full color intensity of spots is reached after about 45 min at room temperature and does not visibly change from then on. The sensitivity of AHTT is below 1 μg of chromatographed palmitaldehyde (Fig. 1) and outranks that of 2,4-dinitrophenylhydrazine (DNPH) spray reagent (0.4 g DNPH + 2 ml concentrated H_2SO_4 + 3 ml H_2O + 10 ml ethanol) (5). During exposure of the chromatogram to air for several hours, the background turns yellow and finally light pink. Autoxidizable lipids produce, during such exposure, color similar to that of aldehydes.

RESULTS

Free aldehyde in lipid mixtures: Five per cent palmitaldehyde was added to fish body lipids and the mixture chromatographed. Figure 2 shows the aldehyde spot detected with AHTT (A), the effect of autoxidation (B), and the chromatogram after heat charring with chromic sulfuric acid (C).

In recent investigations on the enzymatic interconversions of fatty acids and alcohols in tissues of gourami fish, the occurrence of intermediary aldehyde was checked. Lipid extract from an incubation of 200 nmol ^{14}C labeled palmitic acid was chromatographed with hexane + diethyl ether, 85:15, as solvent. AHTT made visible a spot of aldehyde (Fig. 3A) which, after charring of the chromatogram, was hardly detectable (Fig. 3B).

For a duplicate chromatogram, the radioactivity of the aldehyde was measured by scraping the colored area into a scintillation vial containing 1 ml H_2O, counting after addition of 15 ml of Aquasol (New England Nuclear, Pilot Chemicals Division, Boston, Mass.) and applying a correction determined by internal standards. According to these measurements, about 7 nmoles of the radioactive palmitaldehyde were present. AHTT did not indicate any aldehyde in the lipids of the tissue preparation. Accordingly, the amount of aldehyde produced by incubation was about 1.5 μg. The procedure also was applied to SiO_2- glass fiber chromatograms (type SA, Gelman Instrument Co., Ann Arbor, Mich.), and radioactive measurements of the spots cut from the chromatograms gave the same results.

Aldehydogenic lipids: Vinyl ethers are stable under alkaline conditions, and, therefore, plasmalogens do not respond to AHTT unless free aldehydes are formed by acid-catalyzed hydrolysis. For example, after the sequence TLC-acidic hydrolysis-TLC (6), aldehyde is detected easily by AHTT on the 2-dimensional chromatogram. The procedure was applied to lipids of ratfish liver which contain neutral

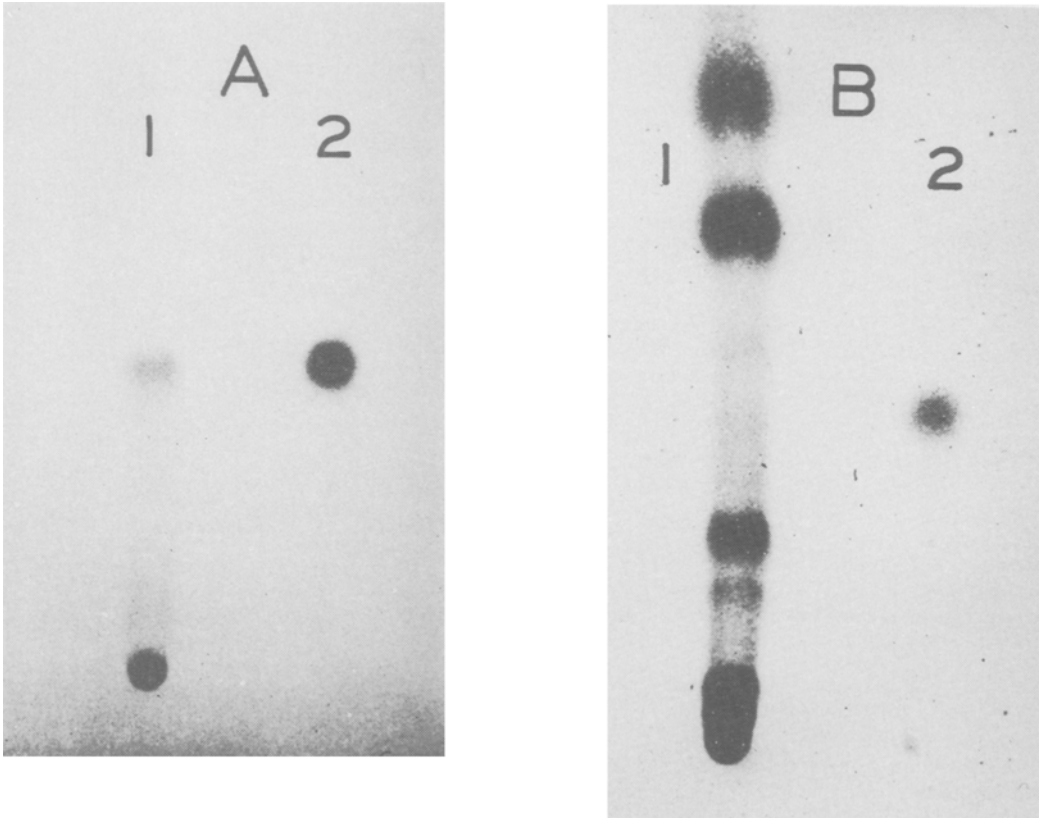


FIG. 3. Chromatogram of (1) lipids containing about 1.5 μg aldehyde and of (2) authentic palmitaldehyde, 45 min after spraying with 4-amino-5-hydrazino-1,2,4-triazole-3-thiol (A) and then heat charred with $K_2Cr_2O_7/H_2SO_4$ (B).

plasmalogens (7). A sample was subjected to TLC on Silica Gel H, twice in the same direction, with petroleum ether (bp 30-60 C) + diethyl ether, 95:5, as solvent. AHTT did not indicate the presence of aldehyde in this chromatogram. A sample was chromatographed as above, exposed to moist HCl vapors, and then chromatographed in the second direction with hexane + diethyl ether, 80:20. Spraying with AHTT revealed the newly formed aldehyde. Corresponding results were obtained with synthetic 1-hexadec-1′-enyl 2,3-di-O-hexadecanoyl glycerol and, under modified chromatographic conditions, with phospholipids and phosphatidyl ethanolamines isolated from gourami fish lipids where both samples contained plasmalogens.

Dimethyl acetal of palmitaldehyde or cyclic acetals of aldehydes with glycerol (8), 1,4-butanediol and 1,3-propanediol, were inert to AHTT, but their aldehyde moiety was readily detected after acidic hydrolysis on the TLC plate. In contrast, vinyl ester bonds, as in octadec-1-enyl acetate are not resistant to alkali and responded to the alkaline spray reagent although slightly slower than free aldehyde.

Ozonization products: Methyl oleate was ozonized in hexane at -70 C and the reaction mixture was chromatographed on Silica Gel H with petroleum ether, (bp 30-60 C) + diethyl ether, 95:5, as developing solvent (9). AHTT made visible spots of several ozonides, as well as the 2 aldehyde fragments which had formed already before development of the plate (Fig. 4A). The aldehyde ester (spot 1) gave a ruby red color different from the purple of the aldehyde (spot 2), and the same was encountered with the aldehyde-acid and aldehyde-alcohol produced from oleic acid and oleyl alcohol, respectively. A somewhat slower staining of the ozonides suggests that they undergo some chemical change, probably forming aldehyde, due to the alkaline solution of AHTT.

AHTT can be used to check the reduction of ozonides to aldehydes. Treatment of the ozonization products with an excess of triphenylphosphine (10) in hexane for 10 min at 0 C increased the amount of aldehydes, but the ozonides had not been split completely (Fig. 4B). However, they were not detected upon

treatment with H_2 over lead-poisoned Pd/$CaCO_3$ catalyst (11) for 10 min under the same solvent and temperature conditions (Fig. 4C).

Specificity: Dickinson and Jacobson claimed specificity of AHTT for aldehyde groups and reported negative test results for esters, ketones, amides, quinones, and other compounds, including formic acid (1). More specifically for lipids, the negative results are confirmed for the usual fatty esters and for ketones, such as methylpentadecyl ketone and 12-ketostearic acid methyl ester.

However, positive reaction of AHTT was encountered with structures that are likely to give rise to aldehyde groups, such as 1-hydroxy-, 1-acetoxy- and 1-bromo-2-ketononadecane, dihydroxyacetone phosphate, and fructose.

AHTT does not react with cholesterol, but stains chromatographed aldosterone and deoxycorticosterone due to their aldehyde and/or terminal ketol groups. 11β-Hydroxyandrosterone and androsterone acetate did not react (all steroids from Schwarz/Mann, Orangeburg, N.Y.). The positive reactions with the former 2 steroids were weaker than with equal amounts of aliphatic aldehydes.

Tests were negative in chromatograms of octadecane, primary fatty alcohols, 1,2-heptadecanediol, 9,10-dihydroxystearic acid, monopalmitins, dihydrophyto- and phytosphingosine, 9,10-epoxystearic acid, palmitoin, oleoin, benzoin, benzil, 16,17-dotriacontadione, glycerol, and inositol. However, when 50 μg or more of such substances were chromatographed in 1 spot, one could detect, after several hours, a very faint pink stain slightly different from the background. When some of the above noncyclic compounds were tested in solution (1), red or purple color eventually appeared. On chromatograms, the differences in time and in intensity of staining when compared to 1 μg of palmitaldehyde rule out interpretation of such compounds as aldehydes.

Chromatography before testing with AHTT for aldehyde is mandatory also with highly unsaturated lipids. Products from inadvertent autoxidation are stained purple like aldehydes, but the chromatogram indicates such condition of the sample. It is advisable to dry chromatograms of unsaturated lipids in a stream of nitrogen, to spray them with the reagent without delay, and to observe the time needed for the development of the stains.

DISCUSSION

When AHTT is allowed to react with aldehyde groups, a purple or red color results. The reaction is very sensitive, (Fig. 1) and, as a spray reagent for chromatograms, AHTT can serve purposes for which mostly DNPH is used. AHTT reacts with aldehydes and 1,2-ketols but in contrast to DNPH does not react with ketones. Furthermore, AHTT does not react with plasmalogens and other aldehydogenic lipids which are stable in alkaline conditions. However, their aldehyde moiety is detected after acidic hydrolysis so that free and bound aldehyde in natural lipids can be distinguished.

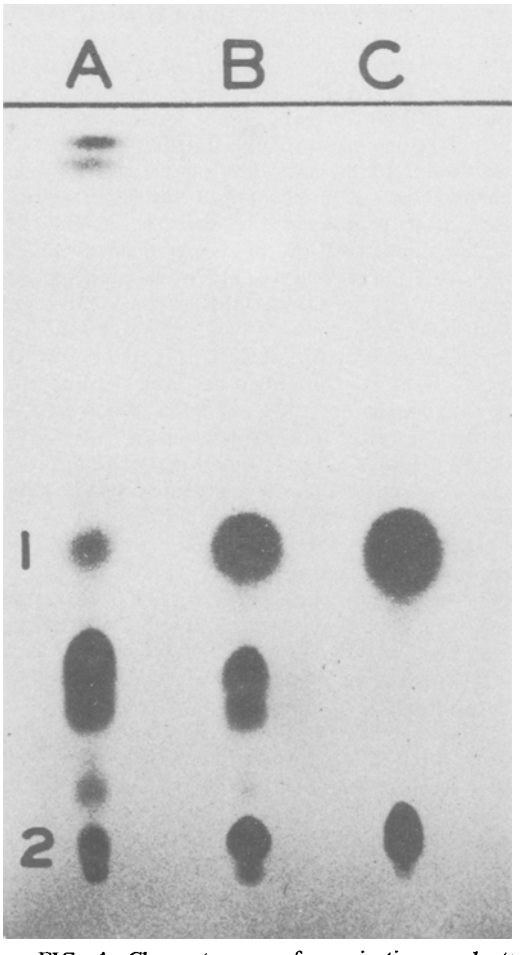


FIG. 4. Chromatogram of ozonization products from methyl oleate (A); an aliquot partially reduced with triphenylphosphine (B); an aliquot completely reduced catalytically with H_2 (C); sprayed with 4-amino-5-hydrazino-1,2,4-triazole-3-thiol. Spot 1, aldehyde; 2, aldehyde-ester.

Advantages of DNPH are its greater stability as a spray reagent and, on larger scale, the preparative use for further chemical analysis of the hydrazones (12). Such application has not been explored yet with AHTT.

The coloration of AHTT with 1-hydroxy-2-

ketones and related structures is likely due to reactions which yield aldehyde groups. Probably for the same reasons, autoxidized lipids react with AHTT, but they chromatograph quite differently from aldehydes so that misinterpretations are unlikely. Autoxidation of unsaturated lipids on developed and sprayed chromatograms brings about the same coloration after prolonged exposure to air (Fig. 2). Under consideration of these points, AHTT indicates the presence of aldehyde in chromatographed lipid mixtures with great certainty and high sensitivity.

In current work, AHTT is applied also to glass fiber chromatograms, and radioactive quantification of ^{14}C labeled aldehydes is carried out after their identification with AHTT on TLC plates (Fig. 3) or on glass fiber sheets. One can expect that colorimetric quantification of aldehydes with AHTT is possible.

AHTT stains red or purple with ozonides, probably due to secondary reactions, and it may be of use when studying the ozonization reaction. Complete reduction of the ozonides can be checked, after chromatography, with AHTT when ozonization is used for structure determination of unsaturated lipids (Fig. 4).

Dickinson and Jacobson discovered that substituted thiourea compounds are converted to AHTT by reaction with hydrazine (4). These authors also recognized the importance of AHTT for detection of aldehydes (1). The results reported here encourage wider practical application of AHTT in conjunction with chromatography for analysis of lipids.

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REFERENCES

1. Dickinson, R.G., and N.W. Jacobsen, J. Chem. Soc. D. 24:1719 (1970).
2. Stolle, R., and P.E. Bowles, Chem. Ber. 41:1099 (1908).
3. Grafe, J., and H. Engelhardt, Chromatographia 5:307 (1972).
4. Dickinson, R.G., and N.W. Jacobsen, Anal. Chem. 41:1324 (1969).
5. Shriner, R.L., R.C. Fuson and D.Y. Curtin, "The Systematic Identification of Organic Compounds, a Laboratory Manual," 5th ed., Wiley & Sons, Inc., New York, 1967, p. 126.
6. Schmid, H.H.O., and H.K. Mangold, Biochim. Biophys. Acta 125:182 (1966).
7. Schmid, H.H.O., W.J. Baumann and H.K. Mangold, Ibid. 144:344 (1967).
8. Baumann, W.J., J. Org. Chem. 36:2743 (1971).
9. Privett, O.S., and E.C. Nickell, J. Lipid Res. 4:208 (1963).
10. Stein, R.A., and N. Nicolaides, Ibid. 3:476 3:476 (1962).
11. Lindlar, H., Helv. Chim. Acta 35:446 (1962).
12. Mahadevan, V., in "Progress in the Chemistry of Fats and Other Lipids," 11:81, esp. pp. 111-114.

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Leaf Wax of Oats[1]

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ABSTRACT

Leaf wax of oats (Kelsey variety) consists of hydrocarbons (5%), esters (10%), free alcohols (45%), free acids (2.5%), β-diketone (5.5%), hydroxy-β-diketones (2.5%), and unidentified (29%). Wax on leaf blades contains more free alcohols than wax on leaf sheaths, and wax on the flag leaf sheath contains more β-diketone than wax on the rest of the plant. Principal hydrocarbons are C_{29}, C_{31}, and C_{33}. The esters, mainly C_{44}-C_{48} and C_{52}, are probably C_{18}-C_{22} and C_{26} esters of hexacosanol. Free alcohols are almost entirely hexacosanol. The β-diketone is hentriacontane-14, 16-dione. Hydroxy β-diketones are a mixture of 5-, 6- and 7-hydroxyhentriacontane-14, 16-diones in the proportions 58:35:7. The wax also contains a small amount (0.5%) of 1,16-hexacosanediol.

INTRODUCTION

During investigations of leaf waxes of plants important in agriculture, compositions of waxes from several wheat varieties were determined (1-3). Leaf wax from oats (*Avena sativa* L. ssp. diffusa (Neils.), Asch. and Graeb., variety Kelsey) has now been analyzed, and the structures of the principal components have been established.

EXPERIMENTAL PROCEDURES

Kelsey variety of oats was grown in the field, and plants were cut when the flag leaf was fully developed and the heads were just emerging; heads were omitted from material extracted as far as possible. Wax was extracted as previously described (1).

Proton magnetic resonance (PMR) spectra were obtained with a Varian HA-100 spectrometer. Natural abundance carbon-13 NMR spectra were measured with a Varian XL-100-15 spectrometer in the Fourier transform mode at 25.2 M Hz with proton noise decoupling (1 K Hz band width). Spectral width was 1000 Hz, aquisition time was 2 *s*, and resolution was 0.075 ppm.

Mass spectra (MS) were measured at the Department of Chemistry, University of Saskatchewan using an MS 12 MS with a direct inlet. Spectra were recorded at 70 ev, with an accelerating voltage of 8 Kv and an ion source temperature of 100 C.

Gas liquid chromatographic (GLC) analyses were performed using a Hewlett Packard Model 402 gas chromatograph with flame ionization detectors. Columns were 3 ft by 1/8 in stainless steel packed with 80-100 mesh, acid washed, and silanized Chromosorb W coated with 1.5% Dexsil 300. Temperature was programed from 125 to 400 C at 3 C/min; flow rate was 60 ml helium/min.

Components were identified by reanalysis after adding synthetic compounds as before (1). Whole wax analyses were performed as follows: wax (ca. 30 mg) was weighed out, and 5 ml of a chloroform solution containing ca. 1 mg each of *p*-dioctylbenzene and octadecyl octadecanoate was added followed by a solution of diazomethane in methylene choride. Solvents were removed, and the mixture was acetylated with acetic anhydride and pyridine (0.5 ml each) at 100 C. Reagents were removed and the sample analyzed as a chloroform solution. Amounts of hydrocarbons were calculated from the response relative to *p*-dioctylbenzene, amounts of alcohols (as acetates) relative to free acids (as methyl esters) and long chain esters were calculated relative to octadecyl octadecanoate. β-Diketones did not give satisfactory peaks after acetylation and were estimated from the UV absorption at 273 nm (4).

Separation of Wax

Wax (11.3 g) was applied to Biosil A (Bio Rad, Richmond, Calif.), (200 g, activated at 100 C for 18 hr), and hydrocarbons (0.97 g) were eluted with hexane (1:1). Hexane-chloroform (90:10, 7 liter) eluted esters (1.05 g), and the same solvents (85:15, 6 liter) eluted a mixture of β-diketone, C_{26}-C_{36} free acids, and unidentified material (0.78 g). β-Diketone (0.32 g) and a mixture of β-diketone and unidentified material (0.28 g) were eluted with hexane-chloroform (80:20, 2 liters, 3 liters). Alcohols (3.55 g) were eluted with hexane-chloroform (75:25, 2 liters), and a mixture of alcohols, free acids, hydroxy β-diketones, and unidentified material (4.30 g) was eluted with the same solvents (1:1, 3 liters) and chloroform (3 liters).

After separation of alcohols (0.79 g) by crystallization from chloroform, the last fraction was treated with diazomethane in methylene chloride and rechromatographed. Methyl esters (C_{16}-C_{26}, 0.15 g) were eluted with

[1]NRCC No. 13472.

hexane-chloroform (85:15, 3 liters), and the same solvents (75:25, 2 liters) eluted alcohols (0.72 g) followed by a mixture of alcohols and hydroxy β-diketones (1.79 g). Hexane-chloroform (1:1, 3 liters) eluted the remainder of the material (0.70 g).

The mixture of alcohols and hydroxy β-diketones was rechromatographed. Elution with hexane-ether (95:5) gave a glyceride-containing fraction (0.2 g) and elution with hexane-ether (90:10, 5 liters) gave alcohols (0.37 g) followed by a mixture of hydroxy β-diketones, alcohols, and unidentified material (0.80 g). All fractions were examined by GLC and thin layer chromatograph (TLC) as before (1,2).

Hydrocarbons

Volatile components, which were determined with triacontane as internal standard, formed only 55% of the fraction; apparently the rest of the material was present as unresolved "chromatographic background" (5).

Esters

Esters (0.05 g) were converted to methyl esters and alcohols by refluxing with 5% methanolic hydrogen chloride (10 ml) and benzene (10 ml) for 18 hr. The mixture of products was acetylated and analyzed by GLC: alcohol acetate peaks had emergence temperatures close to those of methyl esters with 1 more carbon atom in the chain.

β-Diketones

β-Diketone (0.64 g) was separated from crude material as the copper complex (4) and shown to be hentriacontane-14, 16-dione as previously described (2). Methyl esters of free acids and free alcohols were identified as described previously (1,2).

Hydroxy β-Diketones

The mixture of isomers was purified by means of the copper complex (4), rechromatographed, and recrystallized from ethyl acetate to give 0.26 g product. The mp was 74-75 C and $[\alpha]_D^{25}$ + 0.66°, $[\alpha]_{546}^{25}$ + 0.72°, $[\alpha]_{436}^{25}$ + 1.10° and $[\alpha]_{365}^{25}$ + 1.62° (c, 4.7 in $CHCl_3$). PMR spectrum (CCl_4): CH of CHOH at δ3.46 (multiplet) and CH of enol form at δ5.32 (singlet). MS: m/e 480 (mol. ion), 462 (-H_2O), 444 (-2 H_2O), 423 (4,5-cleavage of 5-OH), 409 (5,6-cleavage of 6-OH), 395 (6,7-cleavage of 7-OH), 394 (5,6-cleavage of 5-OH + H), 393, 380 (6,7-cleavage of 6-OH + H), 379, 377 (409-32), 376 (394-H_2O), 366 (7,8-cleavage of 7-OH + H), 364, 363 (395-32), 309, 296 (McLafferty rearrangement), 281 (13,14-cleavage), 269 (16,17-cleavage), 266, 251 (269-H_2O), 239 (15,16-cleavage), 209, 208, 205, 190, 100, 85, 71, 69, 57, 55, 43 (base peak).

Hydroxy β-diketone (0.17 g) was refluxed with 4% ethanolic potassium hydroxide (25 ml) for 18 hr and acidic (0.10 g) and ketonic (0.08) products separated. Acids were treated with diazomethane and separated on a silicic acid column. Methyl palmitate (0.046 g) was eluted with hexane-ether (98:2) and hydroxytetradecanoates (0.044 g) were eluted with hexane-acetone (96:4). Methyl palmitate gave palmitic acid on hydrolysis, mp, and mixed mp 62-63 C.

Hydroxytetradecanoates had $[\alpha]_D^{25}$ + 0.48°, $[\alpha]_{546}^{25}$ + 0.70°, $[\alpha]_{436}^{25}$ + 1.26°, $[\alpha]_{365}^{25}$ + 1.50° (c, 3.6 in $CHCl_3$). PMR spectrum in CCl_4: terminal CH_3 multiplet centered at 0.90; in quinoline: terminal CH_3 2 partly resolved multiplets centered at 0.86 and 0.92. C-13 spectrum is in Table I. MS: m/e 258, 240, 227, 201, 187, 173, 172, 169 (base peak), 158, 155, 144, 143, 141, 87, 74, 69, 55. The ratio of peaks at 169:155:141 was 55:35:10.

The ketonic fraction was separated in the same way and gave C_{17} methyl ketone (0.045 g); mp and mixed mp with heptadecan-2-one was 47-48 C and hydroxy C_{15} methyl ketones (0.042).

Methyl 10-Hydroxytetradecanoate

6-Oxodecanoic acid was coupled anodically with excess methyl hydrogen adipate (6) and after removal of most of the dioxo C_{18} by-product by crystallization, the mixture of 10-oxo C_{14} acid and dimethyl sebacate was reduced with sodium borohydride in methanol. Pure methyl 10-hydroxytetradecanoate was obtained by chromatography on a silicic acid column (elution with hexane-ether 85:15). The yield was 20%. After crystallization from hexane the mp was 35.5-36 C. C-13 spectrum is in Table I. MS: 258, 241, 240, 227, 201, 173, 172, 170, 169 (base peak), 143, 129, 87, 74, 69, 55, 41. $C_{15}H_{30}O_3$ calculated: C 69.72, H 11.70. Found: C 69.68, H 11.63.

Methyl 9-Hydroxytetradecanoate

The hydroxy ester was prepared, as above, from the product of coupling 7-oxododecanoic acid with methyl hydrogen succinate; the yield was 20%. The mp, after hexane crystallization, was 35-35.5 C. C-13 spectrum is in Table I. MS: m/e 258, 240, 227, 208, 188, 187, 158, 155 (base peak), 87, 83, 74, 55. $C_{15}H_{30}O_3$ calculated: C 69.72, H 11.70. Found: C 70.22, H 11.81.

Methyl 8-Hydroxytetradecanoate

Anodic coupling of 6-oxododecanoic acid

TABLE I

Carbon-13 Signals[a] of Methyl Hydroxytetradecanoates

10-Hydroxy	9-Hydroxy	8-Hydroxy	Mixture from hydrolysis of hydroxy β-diketones
14.0 (14)	14.0 (14)	14.0 (14)	14.0
	22.6 (13)	22.6 (13)	22.6
22.7 (13)			22.7
	25.3 (11 or 7)		25.3
		25.4 (6 or 10)	25.4[b]
25.6 (8)	25.6 (7 or 11)	25.6 (10 or 6)	25.6
27.8 (12)			27.8
		31.8 (12)	31.8[b]
	31.9 (12)		31.9
37.1 (11 or 9)			37.1
		37.3 (7 or 9)	
37.4 (9 or 11)	37.4 (8 or 10)		37.4[c]
	37.5 (10 or 8)	37.5 (9 or 7)	

[a]In ppm downfield from tetramethylsilane, carbons to which the signals have been assigned in parentheses, signals due to C_3 (24.9) and C_2 (34.0) and unassigned signals between 29.0 and 29.6 omitted.

[b]Very small signal.

[c]Line width was greater than that of the other signals probably because signals due to C_8 and C_{10} of the 9-hydroxy isomer were not resolved from signal due to C_9 or C_{11} of the 10-hydroxy isomer.

with methyl hydrogen succinate, followed by reduction and purification as above, gave the hydroxy ester in 20% yield. After hexane crystallization the mp was 34.5-35 C. Carbon-13 spectrum is in Table I. MS: m/e 258, 240, 227, 208, 173, 144 (base peak), 141, 101, 95, 87, 74, 69, 55. $C_{15}H_{30}O_3$ calculated: C 69.72, H 11.70. Found: C 69.82, H 11.70.

A mixture containing 10-OH C_{14} 55%, 9-OH C_{14} 38%, 8-OH C_{14} 7% had MS: m/e 258, 240, 227, 208, 201, 187, 173, 172, 170, 169, 158, 155, 144, 143, 141, 129, 115, 109, 101, 87, 83, 81, 74, 69, 67, 55, 43, 41 (base peak). The ratio of peaks at 169:155:141 was 49:43:8. The carbon-13 spectrum of the mixture closely resembled that of the hydroxy C_{14} ester mixture from the hydroxy β-diketones in Table I.

1,16-Hexacosanediol

Material remaining after separation of crude hydroxy β-diketones as the copper complex and material eluted from the column after hydroxy β-diketone (0.93 g) was acetylated and chromatographed on silicic acid. Crude diol diacetate (0.12 g) was eluted with hexane-ether (95:5), and according to GLC 1 component formed 70% of this fraction. Deacetylation and crystallization from chloroform gave pure diol (0.032 g) with mp 97-97.5 C and $[\alpha]_D^{25}$ -0.14°, $[\alpha]_{546}^{25}$ -0.21°, $[\alpha]_{436}^{25}$ - 0.70°, $[\alpha]_{365}^{25}$ - 1.29° (c, 1.4 in pyridine). Oxidation with chromic acid at 100 C (7) gave C_{15} and C_{16} dicarboxylic acids (identified by GLC comparison with authentic acids [8]). MS: m/e 398, 380, 362, 258, 257 (base peak), 239, 221, 171 (CHOH $(CH_2)_9CH_3$), 170, 152, 137, 125, 123, 111, 109, 97, 95, 83, 69, 57, 55, 43, 41. The diacetate prepared from pure diol had PMR spectrum (CCl_4): δ4.00 (triplet, 2 protons, $-CH_2OA_c$) and δ4.80 (multiplet, 1 proton, -CHOAc).

1,16-Pentacosanediol

13-Oxodocosanoic acid (9) was coupled anodically with methyl hydrogen glutarate and the resulting oxo acid reduced with lithium aluminum hydride in tetrahydrofuran. After crystallization from chloroform the yield was 32% and the mp 92-93.5 C. MS: m/e 384, 366, 348, 257 (70% of base peak), 239, 221, 157 ($CHOH(CH_2)_8CH_3$), 156, 137, 125, 123, 110, 109, 97, 95, 85, 83, 69, 57, 55 (base peak), 43, 41. $C_{25}H_{52}O_2$ calculated: C 78.05, H 13.63. Found: C 77.55, H 13.78. GLC comparison of the diacetate with diacetate of the C_{26} diol indicated that the synthetic compound contained 1 less carbon atom.

RESULTS AND DISCUSSION

Composition of wax from whole plants is shown in Table II. Free alcohols were the major components followed by much smaller amounts of esters, hydrocarbons, and β-diketones. Total β-diketones were only 8%; but the absorption at 273 nm indicated 14% β-diketones (assuming

TABLE II

Composition of Leaf Wax of Oats[a]

Component	%
Hydrocarbons	5
Esters	10
Free alcohols	45
Free acids	2.5
β-Diketone	5.5
Hydroxy β-diketones	2.5
1,16-Hexacosanediol	0.5
Glycerides	0.5
Unidentified fractions	
Eluted between esters and β-diketone	4
Eluted between β-diketone and alcohols	5
Eluted after hydroxy β-diketone	20
Yield (% dry wt.)	0.37
$E^{1\%}_{1\,cm}$ at λmax 273 nm (isooctane)	37

[a]Calculated from weights of components obtained by silicic acid chromatography.

$E^{1\%}_{1\,cm}$ = 260 for pure β-diketone), and part of the absorption at 273 nm must be due to other components.

Table III shows the variation in composition of wax from different parts of the plants, β-diketone contents are probably too high, but the relative amounts should be indicated. As was found with wheat plants (10), the proportion of alcohols in wax from leaf blades was higher than it was from leaf sheaths. β-Diketones were major components of wax from the flag leaf sheath, and alcohol content was correspondingly small.

The composition of wax components from whole plants is shown in Table IV. Major hydrocarbons were C_{29}, C_{31}, and C_{33}; hydrocarbons from separate leaves and sheaths were similar but in some cases $C_{29} > C_{31}$. Wheat hydrocarbons contained smaller percentages of C_{33} (1-3). Combined acids were mainly C_{18}-C_{22} with some C_{26}. C_{26} was the major combined alcohol. Thus the major C_{44}-C_{48} and C_{52} esters are probably hexacosyl esters of the C_{18}-C_{22} and C_{26} acids. Free acids differ from combined acids in having much larger amounts of C_{26}-C_{36} acids. Free alcohols, the major wax components, were almost entirely hexacosanol. Oat leaf wax is, therefore, a good source of practically pure hexacosanol. Octacosanol was the major free alcohol of wheat leaf waxes (1-3). There was no evidence for the presence of *trans* 2,3-unsaturated C_{22} and C_{24} acids which were found in wheat leaf waxes. Esters from leaf blades and leaf sheaths were similar to those of wax from whole plants except that there were varying amounts of C_{42} ester. Free alcohols had the same composition from all parts of the plant. Again, as in wheat (1-3), the only β-diketone was hentriacontane-14,16-dione.

Hydroxy β-diketones were a mixture of isomeric hydroxy hentriacontane-14,16-diones. While this investigation was in progress, Dierickx and Buffel (11) reported that wax from caryopses of oat variety Seger I de Svalov (usually known as Victory in North America) contained 3 hydroxy β-diketones 5-(20%), 6-(50%), and 7-(30%) hydroxyhentriacontane-14,16-diones. The hydroxy β-diketones were identified, and the relative proportions of the isomers were estimated, solely from the MS. MS presumably indicates the structure of the isomers but may not give their relative proportions accurately.

In the present investigation the hydroxy β-diketones were investigated in other ways as well as by MS, and the same 3 isomers were found, but in quite different proportions: 5-OH 58%, 6-OH 35%, and 7-OH 7%. The difference in composition from that reported (11) may be due to varietal differences or to wax from caryopses having a composition different from wax from the rest of the plant. MS examination gave peaks at m/e 409 and 394 (5-OH), 409 and 380 (6-OH), and 395 and 366 (7-OH), these 2

TABLE III

Composition of Leaf Wax of Oats from Different Parts of the Plant[a]

Part of plant	Hydrocarbons	Esters	Free alcohols	β-Diketones[b]
Leaf 1 (flag)[c]	7	9	58	12
2	6	8	60	9
3	6	8	64	3
Sheath 1	3	5	8	58
2	5	6	24	10
3	5	7	29	9
Whole plants	4	11	40	14

[a]Wt % determined by gas liquid chromatography.
[b]From $E^{1\%}_{1\,cm}$ at 273 nm assuming pure β-diketone = 260.
[c]Leaves numbered with last formed (flag leaf) as 1.

TABLE IV

Composition of Wax Fractions from Oats[a]

Number of C atoms	Hydrocarbons	Esters	Hydrolysis products of esters		Free acids	Free alcohols
			Acids	Alcohols		
14	—	—	—	—	1	—
16	—	—	5	—	4	—
18	—	—	28	—	2	—
20	—	—	30	—	1	—
21	1	—	—	—	—	—
22	—	—	20	8	6	1
23	1	—	—	—	—	—
24	—	—	4	5	5	—
25	5	—	—	—	—	—
26	—	—	11	83	39	96
27	5	—	—	—	—	—
28	—	—	1	2	11	2
29	23	—	—	—	—	—
30	—	—	1	1	6	1
31	32	—	—	—	—	—
32	—	—	—	—	8	—
33	23	—	—	—	—	—
34	—	—	—	—	9	—
35	6	—	—	—	—	—
36	—	—	—	—	4	—
38	—	1	—	—	—	—
40	—	2	—	—	—	—
42	—	8	—	—	—	—
44	—	31	—	—	—	—
46	—	24	—	—	—	—
48	—	18	—	—	—	—
50	—	4	—	—	—	—
52	—	10	—	—	—	—
54	—	1	—	—	—	—
Unidentified[b]	4 (5)	1 (1)	—	1 (1)	4 (5)	—

[a]From whole plants, wt% determined by gas liquid chromatography.

[b]Number of components in parentheses.

very small. The intensities of the peaks were, however, not reproducible. While 5-OH was certainly the major component, it was not clear whether 7-OH was present or not.

Alkaline hydrolysis and separation into ketonic and acidic fractions and conversion of acids to methyl esters gave methyl palmitate and heptadecan-2, with 1 showing that the parent dione was a 14,16-dione (also indicated by MS peaks 281 $[COCH_2CO[CH_2]_{14}CH_3]$ and 239 $[CO[CH_2]_{14}CH_3]$). Other hydrolysis products were hydroxy C_{15} methyl ketones, which were not investigated further, and a mixture of methyl hydroxytetradecanoates. The PMR spectrum of the hydroxy esters, in quinoline, had terminal CH_3 signals at 0.86 and 0.92 ppm suggesting that ca. 50% of the mixture consisted of 10-OH C_{14} (CH_3 separated from CHOH by 3 methylene groups [12]) derived from 5-OH β-diketone.

Since investigation of carbon-13 NMR spectra of long chain hydroxy esters had shown that many of the signals of isomeric esters are distinguishable (A.P. Tulloch unpublished work), methyl 8-, 9-, and 10-hydroxytetradecanoates were synthesized by standard procedures (6) and carbon-13 spectra measured (Table I). Carbon-13 signals of the mixed hydroxytetradecanoates from the hydroxy β-dikestone also are listed in Table I. Signals were assigned from the carbon-13 spectra of alcohols (13). Since the isomers have similar structures and since all signals in Table I (except C-14) are due to methylene carbons, nuclear Overhauser effect differences are probably small, and it should be possible to estimate the relative proportions of the isomers from the signal intensities fairly accurately.

A mixture of the 3 synthetic isomers was prepared with the composition 10-OH 55%, 9-OH 38% and 8-OH 7% and comparison of the intensities of the signals due to carbon-12 in each isomer gave the composition 10-OH 51%, 9-OH 42%, and 8-OH 7%. Estimation of isomers in the mixture from the hydroxy β-diketone gave 10-OH 58%, 9-OH 35%, and 8-OH 7%.

MS of the hydroxy esters contained peaks expected from the spectra of corresponding

C_{18} hydroxy esters (14), thus 10-OH gave major peaks at 201 (10,11 cleavage), 172 (9,10 cleavage + H) and 169 (201-CH_3OH). Comparison of intensities of peaks at 169, 155, and 141 in MS of the prepared mixture gave 10-OH 49%, 9-OH 43%, and 8-OH 8%. MS of the mixture from the hydroxy β-diketone confirmed that it was a mixture of the 3 isomers and suggested the composition 10-OH 55%, 9-OH 35%, and 8-OH 10%. MS of hydroxytetradecanoates is clearly a more accurate measure of relative proportions than MS of the parent hydroxy β-diketone.

Like the hydroxy β-diketone the mixture of hydroxytetradecanoates was dextrorotatory suggesting the L-configuration, the rotation was similar to that of the 6- and 7-hydroxytetradecanoates from hydroxy β-diketone from club wheat wax (1) and spring wheat wax (3) and also to that of 10-hydroxyhexadecanoic acid from hydroxy β-diketone from durum wheat wax (2).

The presence of 3 isomers raises interesting biosynthetic possibilities, perhaps favoring formation by direct hydroxylation. Microbiological hydroxylation can occur at 2 adjacent positions on a fatty acid chain (15). Though when the positions of the hydroxyl groups are counted from the carbonyl group, in the present hydroxy β-diketone, the principal isomers have hydroxyl groups at positions 9 and 10 and in the hydroxy β-diketone from club and spring wheats (1,3). They are at positions 6 and 7, both pairs of positions being those at which double bonds are usually found.

The PMR spectrum of a fraction eluted after the main alcohol fraction contained the 8 line signals characteristic of protons on C-1 and C-3 of glycerides (16), but pure components could not be isolated. Glycerides were apparently a very minor component of wax from Manitou Wheat (3).

When the relatively polar material, eluted after hydroxy β-diketones, was acetylated and rechromatographed, ca. 0.5% of hexacosane-1,16-diol was isolated as the diacetate. The PMR spectrum showed the presence of acetylated primary and secondary OH groups. After deacetylation, oxidative cleavage (7) gave C_{15} and C_{16} dicarboxylic acids showing that the secondary hydroxyl group was at C-16. MS showed a molecular weight of 398 and a base peak at m/e 257 corresponding to 16,17 cleavage. Synthetic pentacosane-1,16-diol also showed a prominent MS peak at 257 and GLC of the diacetates showed that diol from oat wax contained 1 more carbon atom in the chain. Diols of this structure do not seem to have been isolated previously, most diols from plant waxes being α,ω-diols (17,18). The diol may be biosynthetically related to hexacosanol, the principal free alcohol.

There were several unidentified fractions: components eluted before (4%) and after (5%) the β-diketones were very complex mixtures (GLC). PMR spectra gave no useful information except to show absence of aldehydes. The major unidentified fraction was a brown gum (20%) remaining after isolation of the diol. Similar material also was isolated from wheat waxes (1-3), and again no components could be identified.

The major differences between leaf wax of oats and of wheat is, thus, in the chain length of free and combined alcohols, C_{26} in oats and C_{28} in wheat and in the positions of the hydroxyl group on the hentriacontane-14,16-dione chain of the hydroxy β-diketones. Both waxes contain the same β-diketone.

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REFERENCES

1. Tulloch, A.P., and R.O. Weenink, Can. J. Chem. 47:3119 (1969).
2. Tulloch, A.P., and L.L. Hoffman, Phytochemistry. 10:871 (1971).
3. Tulloch, A.P., and L.L. Hoffman, Phytochemistry. 12:2217 (1973).
4. Horn, D.H.S., Z.H. Kranz and J.A. Lamberton, Aust. J. Chem. 17:464 (1964).
5. Benešová, V., M. Streibl, H.M. Châu, I. Beneš and K. Konečny, Collect. Czech. Chem. Commun. 37:3090 (1972).
6. Tulloch, A.P., JAOCS 41:833 (1964).
7. Tulloch, A.P., Can. J. Chem. 43:415 (1965).
8. Tulloch, A.P., and J.F.T. Spencer, JAOCS 43:153 (1966).
9. Tulloch, A.P., J.F.T. Spencer and M.H. Deinema, Can. J. Chem. 46:345 (1968).
10. Tulloch, A.P., and L. Hoffman, Phytochemistry, 12:2225 (1973).
11. Dierickx, P.J., and K. Buffel, Phytochemistry, 11:2654 (1972).
12. Tulloch, A.P., JAOCS 43:670 (1966).
13. Roberts, J.D., F.J. Weigert, J.I. Kroschwitz and H.J. Reich, J. Amer. Chem. Soc. 92:1338 (1970).
14. Ryhage, R., and E. Stenhagen, Ark. Kemi. 15:545 (1960).
15. Tulloch, A.P., J.F.T. Spencer and P.A.J. Gorin, Can. J. Chem. 40:1326 (1962).
16. Hopkins, C.Y., in "Progress in Chemistry of Fats and Other Lipids," Vol. VIII, Edited by R.T. Holman, Pergamon Press, Oxford, 1965, p. 213.
17. Eglinton, G., and R.J. Hamilton, Science 156:1322 (1967).
18. Tulloch, A.P., Lipids 6:641 (1971).

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Effect of Oxidation on Chemical Spectral and Immunochemical Properties of Egg Yolk Lipoprotein

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ABSTRACT

Studies are reported on the changes in the chemical, spectral, and immunochemical properties of the low density lipoprotein fraction of hen's egg yolk during oxidation. Sonication for 15 min at 15-20 C in the presence or absence of iron gave an increase in thiobarbituric value and UV absorption but did not influence the fluorescence. Storage at 50 C in the absence of iron after sonication for 15 min at 15-20 C produced increases in absorptivity in the fluorescence and UV spectra, as well as thiobarbituric values. Similar changes were produced without sonication in the presence of iron in ca. 8 days storage at 50 C. Ethylenediaminetetraacetic acid completely inhibited the effect of copper, as well as iron, under these conditions. Immunoelectrophoresis pattern was effected by oxidation under conditions that gave an increase in absorptivity in the fluorescence spectra. The first change appeared to involve a minor fast moving component which decreased even when oxidation was inhibited by ethylenediaminetetraacetic acid. As the oxidation proceeded, the mobility of the major component in the immunoelectrophoresis analysis was altered. The major reaction affecting the immunochemical properties appeared to be between the product of lipid oxidation and the protein moiety.

INTRODUCTION

The deterioration of lipoprotein generally is accompanied by changes in the UV absorption spectrum (1) and the ultracentrifugal patterns (2,3). Increase in absorptivity in the fluorescence spectra in the autoxidation of mitochondria and microsomes (4) is also indicative of deterioration of lipoprotein. Sato et al. (5) demonstrated that sonication caused changes in the immunochemical properties of human serum lipoprotein. The deterioration of lipoprotein could occur from an effect on the lipid moiety which, because of its generally high degree of unsaturation, is highly susceptible to autoxidation. The protein moiety also could be altered directly or by interaction with components of the oxidized lipid moiety. To define more clearly the effect of autoxidation on the properties of lipoproteins, immunological, as well as chemical, analyses were carried out on the low density lipoprotein fraction (LDF) of hen's egg yolk oxidized under various conditions.

METHODS

LDF from hen's egg yolk was prepared according to the method of Evans et al. (6).

Lipid class (6) and fatty acid composition analyses were carried out on the lipid extracted from lyophilized LDF with a 1:1 (v/v) mixture of chloroform-methanol. The fatty acid composition was determined on methyl esters prepared by interesterification with methanol-HCl with a gas liquid chromatograph (GLC) (GC-2C,

TABLE I

Lipid Composition of Low Density Lipoprotein Fraction

Composition	% (w/w)
Protein	11.4
Lipids	88.6
Lipids	
Hydrocarbon	0.1
Sterol esters	0.9
Free sterol	14.2
Triglyceride	47.2
Mono- and diglycerides	1.9
Phosphatidyl choline fraction	19.4[a]
Phosphatidyl ethanolamine fraction	11.3[b]

[a]Fraction eluted by methanol-chloroform-water (75:20:5, v/v/v).

[b]Fraction eluted by chloroform-methanol (70:30).

TABLE II

Fatty Acid Composition of Low Density Lipoprotein Fraction

Fatty acid	% (v/w)
16:0	35.3
16:1	4.9
18:0	8.3
18:1	40.9
18:2	10.5

TABLE III

Influence of Sonication Time on Thiobarbituric Acid (TBA) Value, Optical Density (OD), and Fluorescence Absorptivity

Sonication (min)	Fluorescence absorptivity of E_m440 nm at E_x360 nm		(TBA) 535 nm		(OD) 233 nm	
	Catalyst[a]					
	None	Fe^{+++}	None	Fe^{+++}	None	Fe^{+++}
0	32.9	30.0	0.50	0.720	0.290	0.323
5	32.0	35.1	0.910	1.540	0.315	0.371
10	31.5	36.8	1.610	2.240	0.385	0.455
15	35.0	37.0	2.520	2.930	0.460	0.520

[a] $FeCl_3$ 10^{-6} M.

Shimazu Co., Kyoto) equipped with a flame ionization detector using a 3 mm x 2.5 m column of 8% EGS on Chromosorb W.

Solutions of LDF were diluted to contain ca. 5 mg/ml in 0.15 M sodium chloride solution. One milliliter of the LDF solution was exposed to air in small test tubes with glass stopper in a water bath at 50-52 C. Then 0.1 ml of the LDF solution was extracted with 3 ml of ether-ethanol mixture (1:3, v/v) by vigorous mixing for 1 min on a vortex mixer. The extracts were separated by centrifugation at 3000 rpm for 5 min. The organic phase was used for spectrophotometric measurement.

UV and fluorescence spectral analyses were determined with Hitachi EPS-3T and MPF-2 A spectrometers (Hitachi Co., Tokyo), respectively. Thiobarbituric acid (TBA) values were determined by the method of Wilbur et al. (7). Anti-LDF antiserum for the immunochemical analyses was prepared by the subcutaneous injection of 5 mg of LDF emulsified with Freund's complete adjuvant (Iatron Co., Tokyo) into rabbits followed by a booster injection after two weeks.

Immunoelectrophoresis was performed with 1.2% agarose gel in barbiturate buffer at pH 8.6 (μ=0.05) for 1 hr at 2 mA/cm, then antiserum was added to the trough. The plate was incubated at 25 C in a moisture box and observations were carried out for 3 or 4 days (8).

Sonication was performed with a 20 Kc Sonicator (Ohtake Works, Co., Tokyo) at 15-20 C for 15 min.

RESULTS

Lipids and fatty acid composition of LDF are shown in Tables I and II, respectively. Sonication at 15-20 C in the presence, as well as the absence, of iron gave an increase in the TBA and UV absorption at 233 nm in the lipid moiety extracted with ether-ethanol. However, there was virtually no effect on the fluorescence spectrum (Table III). Under more drastic conditions, that is, storage at 50 C after sonication at 15-20 C, changes were produced

TABLE IV

Spectral and Chemical Changes of Low Density Lipoprotein Fraction by Sonication Followed by Long Range Storage at 50 C

Procedure of analysis	Sonication time (min)	Storage at 50 C for		
		0 hr	22 hr	44 hr
Fluorescence absorptivity of E_m440 nm at E_x360 nm	0	11.0	54.2	147.5
	5	10.4	54.4	148.8
	10	12.5	74.9	150.1
	15	11.8	67.0	144.6
Thiobarbituric acid 535 nm	0	0.040	2.910	3.220
	5	0.213	2.670	3.280
	10	0.305	3.180	3.160
	15	0.356	2.630	3.060
Optical density 233 nm	0	0.234	0.810	1.450
	5	0.279	0.690	1.420
	10	0.318	0.880	1.420
	15	0.320	0.785	1.420

in the fluorescence, as well as the UV spectra and TBA values (Table IV).

When the oxidation was catalyzed by iron, similar changes were observed at 4-6 C, as well as at 50 C (Table V).

Immunoelectrophoresis pattern (Fig. 1) showed that the native LDF fraction of hen's egg yolk consisted of two or three components of different mobilities. The faster moving components appeared to be altered first by the effects of oxidation.

As oxidation progressed, the mobility of the major component also was changed, as indicated by immunoelectrophoresis analysis. Copper was used in this experiment because it was less active than iron and showed the sequence of changes better. Ethylenediaminetetraacetic acid (EDTA) inhibited the effect of oxidation catalysts on the chemical changes and the immunochemical properties of the major component of the LDF, but it did not eliminate the effect on the faster moving component.

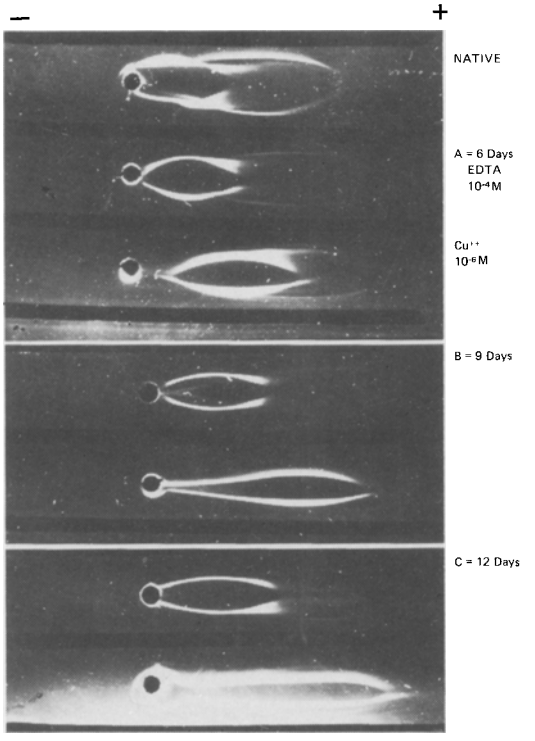

FIG. 1. Immunoelectrophoresis of LDF in the course of storage at 50 C. Native LDF was the sample of the first day of storage without copper and EDTA.

DISCUSSION

The present study showed that LDF of lipoprotein of hen's egg yolk was much more resistant to alteration than the LDF of human serum (5). However, it was evident that, by the use of an oxidation catalyst, copper or iron, similar changes were produced. The difference in resistance of these lipoproteins to alteration appears to be due to ease with which the lipid moiety is oxidized, the LDF fraction of human serum being more unsaturated (9).

The immunochemical analysis showed that oxidation also caused changes in the protein moiety of the lipoprotein. It appears that this change occurs largely as a result of an interaction of the protein with oxidized lipid, inasmuch as the changes in the immunochemical properties appear to be associated with the increase in absorptivity in the fluorescence spectrum. It has been shown that fluorescence products are produced by an interaction of malonaldehyde and amino group (10). Recently Bidlack et al. (11) showed that fluorescent products were formed in peroxide damaged membranes. Inasmuch as the oxidation of LDF

TABLE V

Changes in Optical Density (A), Thiobarbituric Acid Value (B) and Fluorescence Absorptivity (C) of Low Density Lipoprotein Fraction during Long Term Storage at Various Temperatures

	Temperature	Catalyst[a]	0 hr	1 day	2 days	4 days	8 days
A	4-6 C	−	0.470	0.469	0.450	0.450	0.498
	4-6 C	+	0.472	0.587	0.670	1.010	1.810
	50-52 C	−		0.498	0.490	0.605	1.280
	50-52 C	+		1.250	1.970	2.040	2.400
B	4-6 C	−	0.240	0.287	0.360	0.155	0.125
	4-6 C	+	0.810	1.218	1.860	1.880	2.840
	50-52 C	−		0.322	0.455	0.490	1.440
	50-52 C	+		2.980	3.720	2.280	1.450
C	4-6 C	−	10.0	16.6	12.8	12.0	13.0
	4-6 C	+	10.5	12.5	10.8	14.5	24.0
	50-52 C	−		12.1	12.1	28.8	135.6
	50-52 C	+		100.2	299.1	415.8	488.2

[a] $FeCl_3$ 10^{-6} M.

of hen's egg yolk lipoprotein produced a strongly positive TBA value, it is plausible that similar reactions take place on the oxidation of this lipoprotein.

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REFERENCES

1. Robison, W.L., and G.I. Nelson, Biochim. Biophys. Acta, 175:448 (1969).
2. Clark, D.A., E.L. Foulds, Jr. and F.H. Wilson, Jr., Lipids, 4:1 (1969).
3. Nichols, A.V., E.L. Coggiola, L.C. Jensen and E.H. Yokoyama, Biochim. Biophys. Acta, 168:87 (1968).
4. Dillard, C.J., and A.L. Tappel, Lipids 6:715 (1971).
5. Sato, J., H. Shimasaki and I. Hara, Lipids, 7:404 (1972).
6. Evans, R.J., S.L. Bandemer, J.A. Davidson, K. Heinlein and S.S. Vaghefi, Biochim. Biophys. Acta, 164:566 (1968).
7. Wilbur, K.M., F. Bernheim and O.W. Shapiro, Arch. Biochem., 24:305 (1949).
8. Kabat, E., and M.M. Meyer, in "Experimental Immunochemistry," Second edition, C.C. Thomas, Springfield, 1961, p. 78.
9. Skipski, V.P.,in "Blood Lipids and Lipoproteins," Edited by G.J. Nelson, John Wiley and Sons, New York, 1972, p. 546.
10. Chio, K.S., and A.L. Tappel, Biochemistry, 8:2821 (1969).
11. Bidlack, W.R., and A.L. Tappel, Lipids, 8:177 (1973).

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Acetylenic Acids of *Alvaradoa amorphoides* Seed Oil[1]

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ABSTRACT

Oil from the seed of *Alvaradoa amorphoides* Liebm. (Simaroubaceae) collected in Mexico contains two acetylenic fatty acids previously unknown in seed oils, 15% of 17-octadecen-6-ynoic and a trace amount of 6-eicosynoic acid. The predominant fatty acid (58%) in the oil is 6-octadecynoic (tariric). Both the Δ6 and Δ9 series of hexadecenoic, octadecenoic, octadecadienoic, and octadecatrienoic acids were found, but only the Δ6 isomer of eicosenoic acid (1.4%) was detected. The mono- and dienoic acids make up about 19% of the total oil. The remainder consists mostly of saturated acids (6.3%). Techniques used in isolation and identification of the acids included thin layer and gas chromatography, IR, UV, NMR and mass spectroscopy, and ozonolysis coupled with gas chromatography.

INTRODUCTION

Initial analyses of the seed oil of *Alvaradoa amorphoides* (Simaroubaceae) revealed an absorption band at 913 cm^{-1} in the IR spectrum of the oil (1). Later analyses showed that the oil was much like *Picramnia* (2) oil but contained one major and at least three minor unknown components. The major and one of the minor unknowns now have been characterized as acetylenic fatty acids, not cited as seed oil constituents in Smith's review of unusual fatty acids in plants (3). A terminal methylene group in the most abundant new compound accounted for the IR band that led us to undertake this investigation.

EXPERIMENTAL PROCEDURES

Methyl esters were prepared with 10% boron

[1]Presented at the 7th Great Lakes Regional American Chemical Society Meeting, Kalamazoo, Mich., June, 1973.

[2]ARS, USDA.

TABLE I

Composition of *Alvaradoa amorphoides* Oil and Fractions

Component	ECL[c]	Mixed esters	Composition (% by GC[a]) Fractions[b] I	II[d]	III	IV[d]	V	VI
14:0	14.0	0.1	2.9					
16:0	16.0	0.8	8.0					0.1
$16:1^{6c}$ + $16:1^{9c}$	16.4	1.7		5.7	1.9			
17:1	17.3	0.1		0.4	0.1			
18:0	18.0	5.1	81.0					0.6
$18:1^{6c}$	18.3	12.3		66.9				
			0.4		17.0		0.4	1.1
$18:1^{9c}$	18.3	2.4		13.1				
$18:2^{6c,9c}$	18.9	0.2				9.8		
							1.4	0.3
$18:2^{9c,12c}$	18.9	1.7				83.2		
$18:3^{6c,9c,12c}$	19.4	0.4					0.3	77.8
$18:3^{9c,12c,15c}$	19.8	0.1					0.3	13.1
$18:2^{6a,17e}$	20.4	15.3				5.7	96.2	2.8
$18:1^{6a}$	19.8	57.6		7.7	76.0			
19:1	19.3	0.1			2.2			
20:0	20.0	0.4	6.7					
$20:1^{6c}$	20.3	1.4		4.1	1.8			
$20:1^{6a}$	21.8	Trace			0.7			
Unknown	21.4	Trace						1.3
Unknown	21.7	Trace						1.6

[a]GC = Gas chromatography.

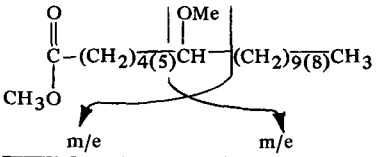
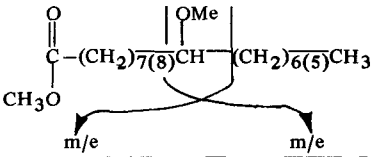
[b]Isolated by preparative thin layer chromatography.

[c]ECL = Equivalent chain length. ECL calculated from LAC-2-R 446 column.

[d]Ratio of 18:1 and 18:2 based upon ozonolysis data.

TABLE II

Fragments from Gas Chromatography–Mass Spectrometry of Methoxylated 16:1 Isomers

Methyl 6- and 7-methoxy palmitate		*Methyl 9- and 10-methoxy palmitate*	
			
m/e	m/e	m/e	m/e
159 173	185 171	201 187	143 129

trifluoride in methanol, according to the method described by Metcalfe et al. (4). A portion of the mixed methyl esters was fractionated by preparative thin layer chromatography (TLC) on 10 x 36 cm plates spread with Silica Gel G containing 20% silver nitrate. The plates were developed with benzene, and the esters were separated into six bands which were observed under UV light after spraying with dichlorofluorescein. Each band (fractions I-VI) was scraped from the plate and extracted with diethyl ether.

The UV spectrum of the mixed esters in ethanol was obtained on a Beckman DK-2A spectrophotometer. IR absorption was measured on solutions of the mixed methyl esters and the preparative TLC fractions in CS_2 and in CCl_4 with a Perkin Elmer Model 137 spectrophotometer using 1 mm NaCl cells. The NMR spectrum of fraction V was obtained in deuteriochloroform containing 1% tetramethylsilane as internal standard with a Varian HA-100 spectrometer.

All gas chromatography (GC) analyses were run on a Packard Model 7401 instrument, as previously described (5,6). Saturated methyl esters of even chain lengths (C_6-C_{22}) were used as standards for the calculations of equivalent chain lengths (ECLs) (7) of ozonolysis products, as well as for long chain fatty esters. The C_{20} monoenoic and monoynoic esters were isolated from fraction III by preparative GC on a 6 ft x 1/4 in. 5% LAC-2-R 446 column. Gas chromatography-mass spectrometry (GC-MS) data were obtained with a Dupont Model 21-492-1 spectrometer in tandem with a Packard Model 7401 chromatograph (8). Positions of unsaturation were determined by ozonolysis coupled with GC (6,9) or by combined GC-MS of methoxy-derivatives (8,10).

RESULTS AND DISCUSSION

GC analysis of the methyl esters prepared from the seed oil of *Alvaradoa amorphoides* indicated the presence of 58% methyl tarirate, 12% methyl petroselinate, and 15% of an unknown component with ECLs suggesting an ene-yne structure (Table I). The UV spectrum of the seed oil did not show any conjugated unsaturation, and the IR spectrum showed no *trans* bonds.

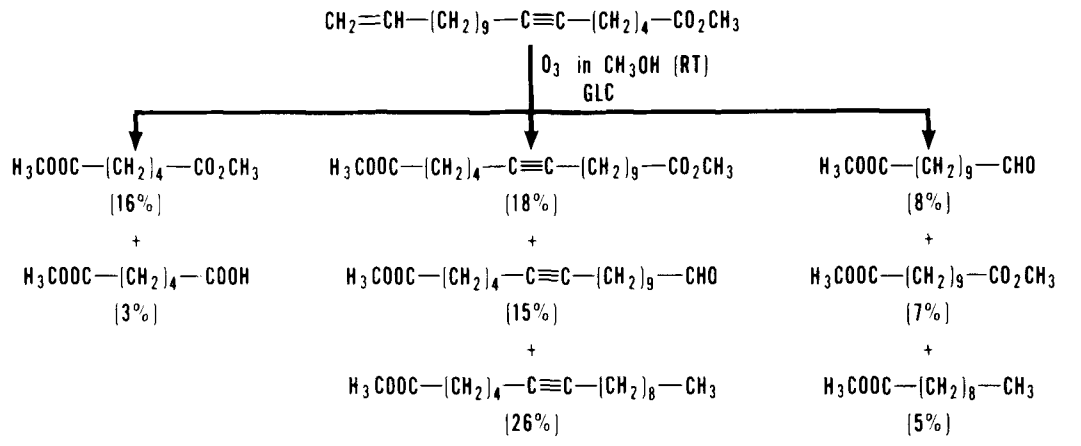
Characterization of Monoenoic Esters

GC of fraction II (Table I) showed these esters to consist mostly of monoenes of various chain lengths with 7.7% monoynoic ester. Ozonolysis of olefinic compounds at low temperature in dichloromethane followed by reduction with triphenylphosphine results in aldehydic fragments (6); under these same conditions, acetylenic bonds are not affected (9). The major fragments found from GC of ozonolysis products of fraction II were C_6 aldehyde-ester (6AE), C_{12} aldehyde (12A), 9AE, and 9A. These products were 87% of the total peak areas and, with their relative proportions, indicate that fraction II has about five times as much 6-18:1 as 9-18:1.

Because of the small amounts of C_{16} and C_{20} esters, procedures were used to characterize them that involved an initial enrichment step. The C_{20} monoene, isolated from fraction III by preparative GC upon ozonolysis yielded 6AE and 14A; only the 6-20:1 was evident from these fragments. The double bond position of the C_{16} monoenes were identified by GC-MS of the methoxy-derivatives of fraction II. The position of the unsaturation is defined by fragmentation adjacent to the methoxy-substituted carbon atoms, as shown in Table II (10). These fragments established the parent esters as 6- and 9-16:1.

Characterization of Monoynoic Esters

Fraction III (Table I) had two esters which migrated on TLC with, or slightly below, monoenoic esters and had ECL of 17.9 and



Scheme I.

19.8 (Apiezon L column) and 19.7 and 21.8 (LAC-2-R 446 column). These characteristics indicated them to be monoacetylenic esters. The rest of fraction III consists of monoenoic esters of various chain lengths (Table I).

Ozonolysis of acetylenic bonds in methanol at room temperature followed by GC yields fragments which are mostly methyl esters with minor amounts of free acids; while under the same conditions, monoenoic esters yield mostly aldehydic fragments with smaller amounts of methyl esters (9). The major ozonolysis fragments from fraction III were 12 ester (E) and six diester (EE); the C_{18} monoynoic ester is, therefore, methyl tarirate. The C_{20} monoynoic ester isolated by preparative GC from this fraction showed the 6EE and 14E from GC of the ozonolysis products. The triple bond is, therefore, in the $\Delta 6$ position. The structures of the acetylenic esters were confirmed by GC-MS.

The spectra of the two monoynoic esters showed an intense peak at m/e 154 (75% relative abundance [RA] in the C_{18} ester and 95% RA in the C_{20} homolog). This peak probably resulted from rearrangement of the acetylene to an allenic structure and cleavage adjacent to the allene ($\oplus C(=O)OCH_3-(CH_2)_4\text{-}CH{=}C{=}CH_2$). A much smaller ion of m/e 140 (RA ca. 10%) also was observed from the MS analysis of both esters. This ion likely results from the cleavage of the molecule after formation of the other possible allene ($\oplus C(=O)OCH_3-(CH_2)_3\text{-}CH{=}C{=}CH_2$). This formation of allenes in the mass spectrometer has been shown by Bohlmann et al. (11) with methyl 6-tetradecynoate (m/e 154) and by Sun and Holman (12) with methyl stearolate (m/e 196 and 210). In addition, the spectra of the esters under investigation showed appropriate molecular ions (C_{18} = 294, C_{20} = 322).

Characterization of Enynoic Ester

Fraction V consisted mostly (96%, Table I) of an ester with ECLs of 19.6 (Apiezon L) and 20.5 (LAC-2-R 446). These ECLs are consistent with those of C_{18} esters with nonconjugated ene-yne systems as found in methyl crepenynate (13) and methyl 17-octadecen-9-ynoate (14).

NMR of fraction V showed three vinyl protons (τ 4.2 and 5.1), six protons alpha to unsaturation (τ 7.85), two protons alpha to the carboxyl group (τ 7.7), three *O*-methyl protons (τ 6.3), and 18 methylene protons (τ 8.7). No protons due to a terminal methyl group (τ 9.1) were found.

The IR spectrum of fraction V showed, besides the bands usually found in long chain fatty esters, absorption at 913, 990, and 1635 cm^{-1}, indicative of an ester with a terminal olefinic bond (14). Both NMR and IR data were in good agreement with those from known methyl 17-octadecen-9-ynoate.

The mass spectrum of the major ester in fraction V was almost identical to that of methyl tarirate. Ions at m/e 154 (RA 75%) and m/e 140 (RA 8%) indicated that the triple bond is in the $\Delta 6$ position. Both the molecular ion (m/e 292) and molecular ion minus methoxyl (m/e 261) were two mass units less than those of methyl tarirate. The spectral evidence indicates the structure of the ene-yne fatty ester as methyl 17-octadecen-6-ynoate. This structure was confirmed by the ozonolysis-GC procedure of Spencer et al. (9). All fragments normally resulting from this reaction were found and

identified by their ECLs from GC (Scheme I). Two additional products were observed from this analysis, methyl 6-hexadecynoate and methyl decanoate. The methyl 6-hexadecynoate most likely results from degradation of methoxy-hydroperoxide formed during ozonolysis in methanol (15). The methyl decanoate presumably is formed by the same mechanism applied to the ozonolysis fragments resulting from cleavage at the triple bond.

The MS of all ozonolysis fragments confirmed the GC identification; in addition, all fragments proposed to contain $\Delta 6$ triple bonds showed an intense ion at m/e 154.

Characterization of Dienoic and Trienoic Esters

Ozonolysis of fraction IV in dichloromethane at dry-ice acetone temperature and subsequent GC (6) yielded fragments that would result from a mixture of 6,9-18:2 and 9,12-18:2, namely, 9A (3.7%), 6AE (3.1%) and 6A (31%), 9AE (43%). The ozonolysis-GC products of fraction VI were consistent with a mixture of 6,9,12-18:3 and 9,12,15-18:3 (6AE and 9AE). The mass spectra obtained for the two trienoic esters in fraction VI were nearly identical with those reported by Holman et al. (16) for methyl linolenate and methyl γ-linolenate.

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R.G. Powell provided methyl 17-octadecen-9-ynoate; D. Weisleder performed NMR analysis; and Q. Jones supplied seed.

REFERENCES

1. Jones, Q., and F.R. Earle, Econ. Bot. 20:127 (1966).
2. Spencer, G.F., R. Kleiman, F.R. Earle and I.A. Wolff, Lipids 5:285 (1970).
3. Smith, C.R., Jr., "Progress in the Chemistry of Fats and Other Lipids," Vol. XI, part 1, Edited by R.T. Holman, Pergamon Press, Oxford, England, 1970, p. 137.
4. Metcalfe, L.D., A.A. Schmitz and J.R. Pelka, Anal. Chem. 38:514 (1966).
5. Kleiman, R., G.F. Spencer, L.W. Tjarks and F.R. Earle, Lipids 6:617 (1971).
6. Kleiman, R., G.F. Spencer, F.R. Earle and I.A. Wolff, Ibid. 4:135 (1969).
7. Miwa, T.K., K.L. Mikolajczak, F.R. Earle and I.A. Wolff, Anal. Chem. 32:1739 (1960).
8. Spencer, G.F., R. Kleiman, R.W. Miller and F.R. Earle, Lipids 6:712 (1971).
9. Spencer, G.F., R. Kleiman, F.R. Earle and I.A. Wolff, Anal. Chem. 41:1874 (1969).
10. Abley, P., F.J. McQuillin, D.E. Minnikin, K. Kusamron, K. Mashers and N. Polgar, Chem. Commun. 1970:348.
11. Bohlmann, F., D. Schumann, H. Bethke and C. Zdero, Chem. Ber. 100:3706 (1967).
12. Sun, K.K., and R.T. Holman, JAOCS 45:810 (1968).
13. Mikolajczak, K.L., C.R. Smith, Jr., M.O. Bagby and I.A. Wolff, J. Org. Chem. 29:318 (1964).
14. Powell, R.G., and C.R. Smith, Jr., Biochemistry 5:625 (1966).
15. Pryde, E.H., and J.C. Cowan, "Topics in Lipid Chemistry," Vol. II, Edited by F.D. Gunstone, Logos Press, London, England, 1971, p. 1.
16. Holman, R.T., and J.J. Rahm, "Progress in the Chemistry of Fats and Other Lipids," Vol. 9, part 1, Edited by R.T. Holman, Pergamon Press, Oxford, England, 1966, p. 13.

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Determination of Molecular Species of Ovolecithin Using Gas Chromatography-Mass Spectrometry

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ABSTRACT

An analysis of monoacetyldiglyceride was performed by gas chromatography-mass spectrometry for the purpose of determining the molecular species of ovolecithin. Separation of monoacetyldiglycerides was made according to the degree of unsaturation and to the sum of the carbon numbers of fatty acyl residues. Fragments of monoacetyldiglyceride were analyzed and interpreted in relation to the molecular structure in a similar manner to that of triglyceride.

INTRODUCTION

The analysis of phospholipids in terms of molecular species has become increasingly more important, along with the recognition of their function in the living cells. The enzymic method using phospholipase C and pancreatic lipase has been employed by many investigators (1-3). This method is elaborate and time consuming. The present paper deals with the analysis by means of gas chromatography-mass spectrometry (GC-MS) of molecular species of the monoacetyldiglyceride (MADG) derived from phosphatidylcholine. The method is applicable to the whole class of glycerophospholipid.

EXPERIMENTAL PROCEDURES

Ovolecithin (phosphatidylcholine from hen egg yolk) was prepared according to the method of Pangborn (4) and purified by DEAE-cellulose chromatography (5). Synthetic samples of dipalmitoyl-phosphatidylcholine and dioleoyl-phosphatidylcholine (Applied Science Co.) were used as authentic standards.

Acetolysis was carried out in a mixture of acetic anhydride and acetic acid (2:3) at 150 C for 5 hr (6). Under these conditions the phosphatidylcholines were converted completely to MADG, giving single spots by thin layer chromatography (TLC) on Silica Gel G plates (petroleum ether-ether, 85:15). The MADG was subfractionated according to the degree of unsaturation by TLC on $AgNO_3$-Silica Gel G (benzene-ether, 8:2) (7). The subfractions obtained were scraped off from the

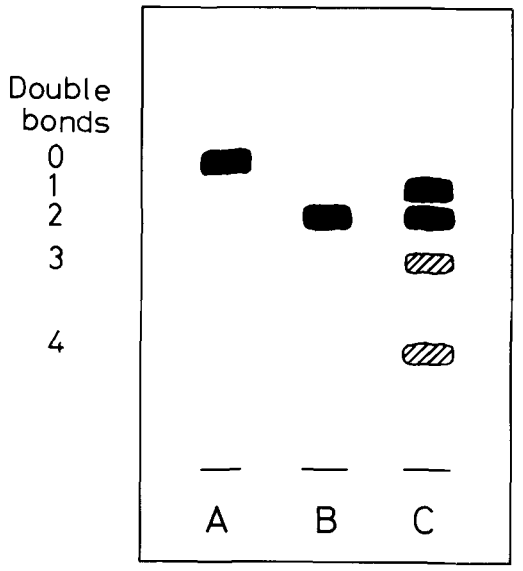


FIG. 1. Thin layer chromatogram of monoacetyldiglyceride (MADG) on $AgNO_3$-Silica Gel G plate. Solvent: benzene-ether (8:2). A. MADG from dipalmitoyl-phosphatidylcholine. B. MADG from dioleoyl-phosphaditylcholine. C. MADG from ovolecithin.

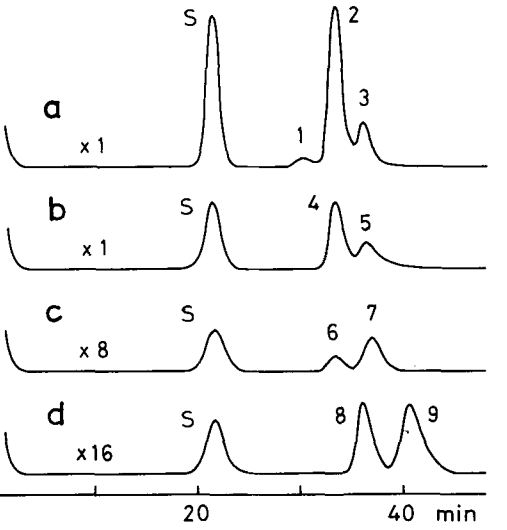


FIG. 2. Gas chromatograms of subfractions of monoacetyldiglyceride (MADG) from ovolecithin. a, b, c, and d: monoenoic, dienoic, trienoic, and tetraenoic MADG, respectively. Peak S: internal standard, tricaprin.

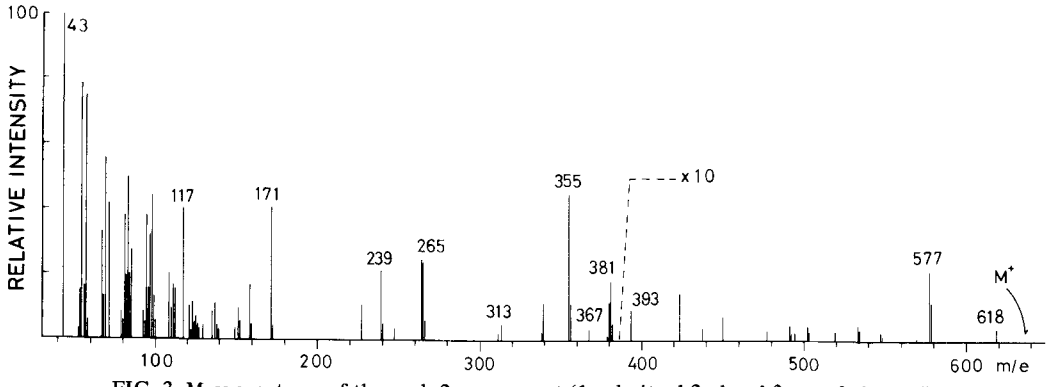


FIG. 3. Mass spectrum of the peak 2 component (1-palmitoyl-2-oleoyl-3-acetyl-glycerol).

TABLE I

Principal Ions in Mass Spectra of Monoacetyldiglyceride (MADG) Derived from Ovolecithin

Fatty acid 1 position	16:0[a]	16:0	16:0	16:0	16:0	18:0	18:0	18:0	18:1[a]	18:1
2 position	16:0	16:1	18:1	18:2	20:4	18:1	18:2	20:4	18:1	18:2
Ion structure	m/e	m/e	m/e	m/e	m/e	m/e	m/e	m/e	m/e	m/e
M^{+}[b]	610	608	636	634	658	664	662	686	662	660
$[M-18]^{+}$	592	590	618	616	640	646	644	668	644	642
$[M-CH_3COO]^{+}$	551	549	577	575	599	605	603	627	603	601
$[M-R^1COO]^{+}$	355	353	381	379	403	381	379	403	381	379
$[M-R^1COOCH_2]^{+}$	341	339	367	365	389	367	365	389	367	365
$[M-R^2COO]^{+}$	355	355	355	355	355	383	383	383	381	381
R^1CO^{+}	239	239	239	239	239	267	267	267	265	265
$[R^1CO+74]^{+}$	313	313	313	313	313	341	341	341	339	339
$[R^1CO+128]^{+}$	367	367	367	367	367	395	395	395	393	393
R^2CO^{+}	239	237	265	263	286	265	263	286	265	263
$[R^2CO+74]^{+}$	313	311	339	337	360	339	337	360	339	337
$[R^2CO+128]^{+}$	367	365	393	391	414	393	391	414	393	391
CH_3CO^{+}	43	43	43	43	43	43	43	43	43	43
$[CH_3CO+74]^{+}$	117	117	117	117	117	117	117	117	117	117
$[CH_3CO+128]^{+}$	171	171	171	171	171	171	171	171	171	171

[a]Dipalmitoyl-MADG and dioleoyl-MADG were derived from synthetic phosphatidylcholine.

[b]M^{+} ion peaks were not detected except in the cases of MADG which contained 20:4 acid.

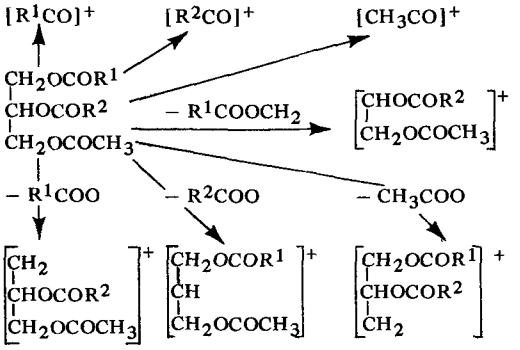
TABLE II

Molecular Species of Monoacetyldiglyceride (MADG) from Ovolecithin

MADG peak	Double bonds	Sum of FA[a]	Fatty acid 1 position	Fatty acid 2 position	Content, %
1	1	32	16:0	16:1	0.6
2	1	34	16:0	18:1	44.4
3	1	36	18:0	18:1	19.7
4	2	34	16:0	18:2	16.6[b]
5	2	36	18:0	18:2	8.6
7	3	36	18:1	18:2	1.8
8	4	36	16:0	20:4	3.1
9	4	38	18:0	20:4	4.9

[a]Sum of carbon numbers of fatty acyl residues.

[b]Content of peak 4 includes that of peak 6 (0.6%). Latter component (peak 6) was identified mass spectrometrically as the same as the former (peak 4).



Scheme 1.

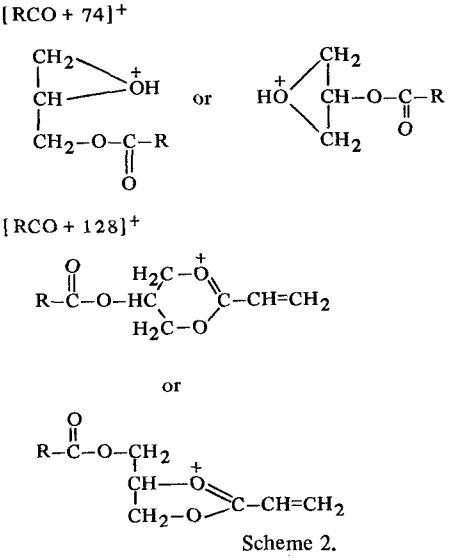
chromatoplate and extracted with chloroform-methanol (2:1). The ratio of the individual subfractions was determined by adding known amounts of tricaprin, as an internal standard, to each fraction.

The subfractions of MADG were fractionated further by gas liquid chromatography (GLC) according to the sum of the carbon numbers of fatty acyl residues (8) and analyzed by mass spectrometry. The GC-MS analysis was carried out on an LKB-9000S gas chromatograph-mass spectrometer, equipped with a 35 cm x 3 mm glass column packed with 2.5% silicone OV-17 on 60-80 mesh Shimalite W. The column oven temperature was programed from 180-280 C at a rate of 3 C/min. The molecular separator was maintained at 310 C, as was the ion source. The ionizing voltage was 70 eV and trap current was 60 μA. Mass spectra were recorded to m/e 750 in a total scan time of 8 sec at the apex of the GLC peak as determined by the continuous record produced by a total ion monitor.

RESULTS AND DISCUSSION

The MADG from ovolecithin were separated into four subfractions, monoenoic, dienoic, trienoic and tetraenoic, by TLC on $AgNO_3$-Silica Gel G (Fig. 1). Each subfraction was further fractionated by GLC according to the sum of the carbon numbers of fatty acyl residues (Fig. 2).

The mass spectra of MADG were interpreted in relation to the molecular structure in a manner similar to that of triglycerides reported by Barber et al. (9) (Scheme 1). The base peak of the spectrum of MADG was in most cases m/e 43, which corresponded to $[CH_3CO]^+$. The fatty acid moieties were identified by the intense peaks corresponding to $[M - R^{1,2}COO]^+$ and $[R^{1,2}CO]^+$ ions. The fatty acid in the 2 position of the glycerol was identified from the fact that $[M - R^2COOCH_2]^+$ peak usually is significantly smaller than $[M - R^1COOCH_2]^+$ peak. The presence of the glycerol skeleton, to which each fatty acid was bound directly, was demonstrated by the two sets of peaks corresponding to $[R^{1,2}CO + 74]^+$ and $[R^{1,2}CO + 128]^+$, aside from $[CH_3CO + 74]^+$ (m/e 117) and $[CH_3CO + 128]^+$ (m/e 171) (Scheme 2) (10). The principal ion fragments of ten MADG are listed in Table I.



Scheme 2.

The mass spectrum obtained from the peak 2 in Figure 2 is illustrated in Figure 3. The component was identified as 1-palmitoyl-2-oleoyl-3-acetyl-glycerol. Table II shows the molecular species of MADG obtained from ovolecithin.

It is known that acetolysis of glycerophospholipids can be carried out without any appreciable intermolecular exchange of fatty acids (6). The formation of 2-acetyl-1,3-diglycerides was not detected in this experiment. Consequently, the intact molecular species of glycerophospholipids are supposed to be the same as those of MADG obtained by the present procedure. The results shown in Table II agreed with the findings in the experiments using phospholipase A, which indicated that more than 98% of the fatty acids bound in the 2 position were unsaturated acids (11).

REFERENCES

1. van Golde, L.M.G., and L.L.M. van Deenen, Chem. Phys. Lipids 1:157 (1967).
2. Lands, W.E.M., and P. Hart, JAOCS 43:290 (1966).
3. van Golde, L.M.G., and L.L.M. van Deenen, Biochim. Biophys. Acta 125:496 (1966).

4. Pangborn, M.C., J. Biol. Chem. 188:471 (1950).
5. Rouser, G., G. Kritchevsky, A. Yamamoto, G. Simon, C. Galli and A.J. Bauman, in "Methods in Enzymology," Vol. 14, Edited by J.M. Lowenstein, Academic Press, New York and London, 1969, p. 272.
6. Renkonen, O., JAOCS 42:298 (1965).
7. Kaufmann, H.P., and H. Wessels, Fette Seifen Anstrichm. 66:81 (1964).
8. Kuksis, A., JAOCS 42:269 (1965).
9. Barber, M., and T.O. Merren, Tetrahedron Lett. 18:1063 (1964).
10. Odham, G., and E. Stenhagen, in "Biochemical Applications of Mass Spectrometry," Edited by G.R. Waller, John Wiley & Sons, Inc., New York and London, 1972, p. 229.
11. Saito, K., and D.J. Hanahan, Biochemistry 1:521 (1962).

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Plasma Cholesterol Levels in Suckling and Weaned Calves, Lambs, Pigs, and Colts

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ABSTRACT

Plasma cholesterol levels were determined in calves, lambs, and pigs at intervals from birth until after weaning. In each case the levels were low at birth, became elevated during the suckling period, and decreased as the animals began to eat solid feed. Results with calves fed skim milk indicated that milk lipids were largely responsible for the post-partum elevation of plasma cholesterol levels. Studies with early and late weaned pigs also indicated that the elevation of plasma cholesterol in suckling animals was related to diet rather than age. Sex and breed had no apparent effect on plasma cholesterol levels in these experiments. A limited number of observations in colts indicated that plasma cholesterol levels decreased between 2 and 7 months.

INTRODUCTION

It is well known that serum cholesterol levels in humans are low at birth and increase quite rapidly during the first few weeks of life (1-3). This appears to be an effect of diet, since the increase largely can be prevented by feeding milk substitutes (3-6). Studies on cattle likewise have shown a marked increase in serum cholesterol during the early postnatal period (7-10). Hypercholesterolemia also has been observed in suckling rats and rabbits (7,11-15) and has been attributed to the cholesterol (12) and to the high fat content (14) of milk.

The level of serum cholesterol decreases rapidly in rats and rabbits after weaning (7,11-15) and a more gradual decrease has been observed in calves (7,10). By contrast, elevated serum cholesterol levels are maintained in humans through childhood, adolescence, and into adult life (16-19). This pattern is not universal, however, since Whyte and Yee (20) found that serum cholesterol levels in children from New Guinea showed a gradual decrease after one year of age.

Because of the observed difference between humans and other animal species and because the factors that influence blood cholesterol levels are still poorly understood, the present experiments were carried out to obtain further information on changes in plasma cholesterols of different species during the early postnatal period. Two species of ruminants (sheep and cattle) and two species of nonruminants (swine and horses) were included in these studies. In addition to measuring plasma cholesterols of animals under regular management, an experiment was performed to compare the effects of whole and skim milk in neonatal calves, and another experiment was carried out to assess the effects of early and late weaning in pigs. A preliminary report of some aspects of this work has been presented (21).

EXPERIMENTAL PROCEDURES

The calves, lambs, pigs, and colts used for these experiments were from herds maintained by the University of Guelph.

Calves – Whole milk-fed: Male or female Holstein calves were nursed by their respective dams until seven days of age, at which time they were transferred to a pail-feeding program of whole milk. The consumption of whole milk was limited to 2.7 kg/day. After 10 days of age the calves had access to pelleted calf starter (containing 20% crude protein and 2.5% crude fat; obtained from Master Feeds Division, Maple Leaf Mills, Ltd., Toronto) and good quality hay. The calves were weaned from milk at 35 days of age.

Skim milk-fed: Male Holstein calves received colostrum from their dams for the first two days after birth, then 50% skim and 50% whole milk for the next two days and skim milk alone from the fifth day until weaning from milk at 35 days of age. The skim milk was reconstituted by adding 11 parts of water to 1 part of skim milk powder. Calf starter and forage were offered as above.

Lambs: Male and female lambs (Suffolk or Southdown x Suffolk) were suckled by their respective ewes and had access to the adults' feed during this period. Castration of the males

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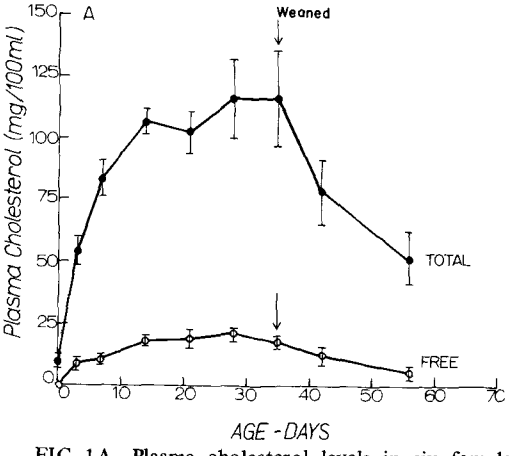

FIG. 1A. Plasma cholesterol levels in six female Holstein calves weaned at 35 days of age. Each point represents mean ± SEM of 4-6 determinations.

and docking of the lambs was performed at 10-14 days of age. All lambs were weaned from their respective dams at 45 days of age.

Pigs–Regular management: Male and female Yorkshire pigs were nursed by their respective sows until they were weaned at 35 days of age. After 7 days the young pigs had access to pig starter containing 20% crude protein, derived from corn, soybean meal, and fish meal.

Special management: Male and female pigs from two breeds, Yorkshire and Lacombe, were suckled by their respective sows but received no pig starter during the suckling period. One group, 12 pigs from each breed, was early weaned at 21 days, while another group of the same number was late weaned at 35 days of age.

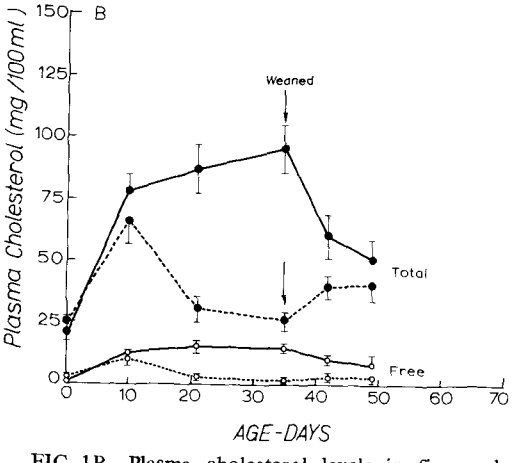

FIG. 1B. Plasma cholesterol levels in five male Holstein calves fed whole milk (———) and six males receiving skim milk (- - - -). The transfer to skim milk was made gradually between 2 and 5 days of age. Mean ± SEM.

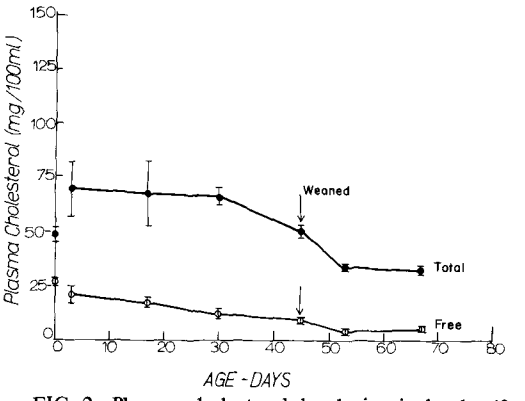

FIG. 2. Plasma cholesterol levels in six lambs (3 males, 3 females) weaned at 45 days of age. Cholesterol levels obtained on the day of birth from a separate group of eight lambs (2 males, 6 females) also are shown. Mean ± SEM.

Irrespective of management, all pigs received injectable iron and male pigs were castrated at ca. 3 weeks of age.

Colts: Colts from both light and heavy breeds were raised by their respective mares and had access to the adults' feed during the suckling period.

Chemical analyses: Blood samples were taken from the jugular vein of calves, lambs, and colts, from the eye socket of new born pigs, and from the vena cava of older pigs. Ethylenediaminetetraacetate (EDTA) was used as anticoagulant. Plasma obtained by centrifugation was stored frozen until the analyses were performed. Total and free cholesterol were measured by the method of Sperry and Webb (22). The same method was used to determine the total cholesterol of colostrum, whole milk, and skim milk. The total lipid content of these samples was determined by the method of the Association of Official Agricultural Chemists (23).

RESULTS

Calves: The plasma cholesterol in female calves was lowest at birth, showed a rapid increase during the first 14 days postpartum, and then leveled off during the next 3 weeks. After weaning, the cholesterol level decreased rapidly to about one-half of the peak level (Fig. 1A).

Another experiment was conducted to assess the effects of the lipid and nonlipid components of milk. The results are shown in Figure 1B.

The pattern with male calves fed whole milk was similar to that observed earlier with female calves. With calves fed skim milk, the choles-

terol levels also increased for the first 10 days postpartum, probably because these calves received colostrum and some whole milk until 5 days of age. After 10 days there was a rapid decrease, and the plasma cholesterols remained significantly lower than in the whole milk-fed animals ($P<0.05$) until the calves were weaned. After weaning the levels converged so that there was no significant difference between the whole and skim milk-fed calves at 42 and 49 days of age. In all of these experiments with calves, the level of free cholesterol followed the same trend as the total cholesterol.

Lambs: A group of 3 male and 3 female lambs was used for these studies and blood samples were taken at intervals from 3 to 67 days of age. Since there was no apparent sex difference in plasma cholesterol levels, the results for both males and females are combined in Figure 2. From 3 to 30 days the plasma total cholesterol remained fairly constant and then decreased between 30 and 53 days of age. Plasma free cholesterol also decreased over this period of time. After 53 days of age, the levels of free and total cholesterol remained fairly constant and were similar to values obtained with four other lambs at 6 months of age.

Blood samples were subsequently obtained from a group of 8 lambs (6 females, 2 males) on the day of birth. As shown in Figure 2, the levels of total cholesterol at birth tended to be lower than those observed in 3 day old lambs, while the free cholesterol was, if anything, higher at birth.

Pigs: Figure 3A shows the plasma cholesterol levels in 6 male and 5 female Yorkshire pigs from four different litters. All pigs were weaned at 36 days and had access to pig starter after 7 days of age. Since there were only minor differences in the cholesterol levels of the male and female pigs, the data were pooled. The total and free cholesterol levels shown for the day of birth were obtained at a later date for a different group of 10 pigs (7 females, 3 males).

The total plasma cholesterol at birth averaged 50 mg/100 ml compared to 135 mg/100 ml at 3 days of age. From 3 to 14 days of age, there appeared to be no change, but after 14 days the level decreased continuously until 42 days of age. The plasma free cholesterol followed the same general trend as the total cholesterol.

Since these pigs received pelleted pig starter during the suckling period, the decrease in plasma cholesterol levels after 14 days of age may have been due to an increased intake of solid feed and a decreased consumption of sow's milk. Therefore, another experiment was

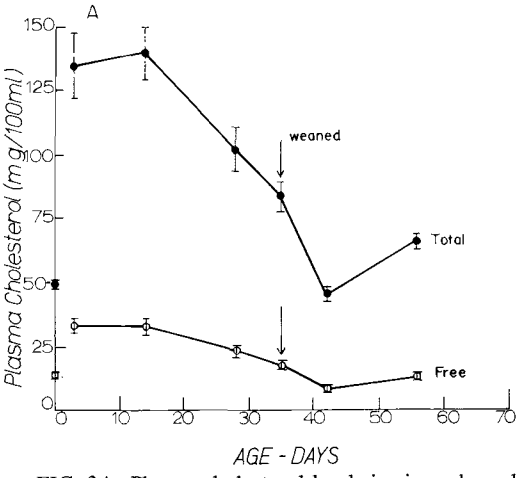

FIG. 3A. Plasma cholesterol levels in six male and five female Yorkshire pigs which had access to pig starter after 7 days and were weaned at 36 days of age. Cholesterol levels shown for the day of birth are from a separate group of 10 pigs (3 males, 7 females). Mean ± SEM.

conducted in which the pigs received no pig starter during the suckling period and were either early weaned at 21 days or late weaned at 35 days of age. Sex and breed had no apparent effect on plasma cholesterol levels, so the results from both males and females of the two different breeds were combined for presentation in Figure 3B. In this experiment, the plasma cholesterol remained elevated in each group until the animals were weaned and then decreased rapidly. This indicates that the decrease observed prior to weaning in the first experiment (Fig. 3A) was related to consumption of pig starter.

Colts: Plasma cholesterol levels in three or four colts from both sexes are shown in Figure 4. The data are rather incomplete, since it was difficult to get blood samples from colts of known ages, but the results indicate a continuous decrease in total cholesterol between 2 and 7 months of age. Plasma free cholesterol also decreased during this period. The age at weaning was not standardized as in the experiments with other species, but generally occurred when the colts were 3-5 months old. These colts had access to the adults' feed during the suckling period.

Milk lipids: The milk fat and cholesterol content of representative samples of colostrum and whole milk from cows, ewes, and sows and of skim milk from cows are given in Table I. The colostrum was characterized by a high cholesterol and total lipid content, except for sows. Whole milk from ewes and sows has a

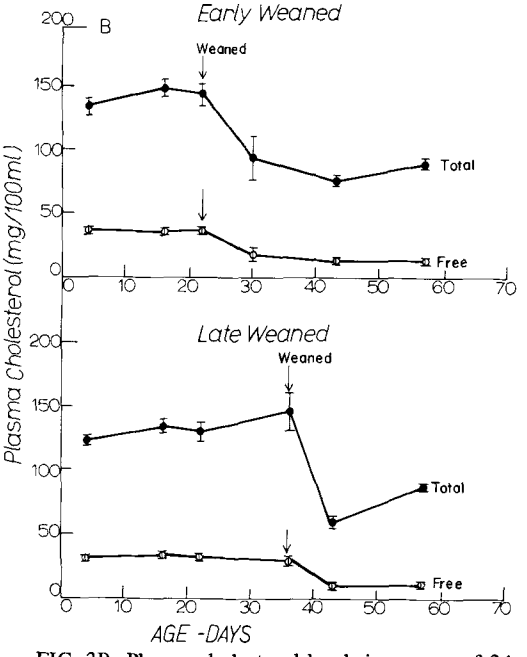

FIG. 3B. Plasma cholesterol levels in groups of 24 pigs which had no access to pig starter during the suckling period and were weaned either at 21 or 35 days of age. Each group contained six male and six female pigs from each of two breeds, Yorkshire and Lacombe. Mean ± SEM.

higher total lipid and cholesterol content than cow's milk. The skim milk had a low lipid content.

DISCUSSION

The results of these experiments with young calves, lambs, and pigs showed changes in levels of plasma cholesterol similar to those observed previously in cattle, rats, and rabbits (6-15). The values were low at birth, increased during the immediate postnatal period and then decreased again as the animals were weaned (Figs. 1-3). Our values for young calves at birth and during the early suckling period agree with those reported in earlier studies (7-10), but in our experiments the levels decreased more quickly after weaning (Fig. 1).

Our results are also in general agreement with other recent studies on calves, lambs, and pigs. Wiggers et al. (24) measured plasma cholesterol levels in calves over a 24-week period, beginning at 4 days of age. The levels rose more or less continuously when the diet consisted of whole milk for the entire period, and considerably higher levels were observed when the milk was supplemented with cholesterol. Calves weaned from milk to grain at 4 weeks of age showed a decrease in plasma cholesterol and the levels remained low on the grain diet. In another recent study, Tumbleson and Hutcheson (25) measured serum cholesterol levels in female dairy cattle ranging from 1 month to 16 years of age. They reported a gradual increase up to 3 years and a decrease thereafter. It is not possible to deduce from their results whether weaning had any effect, since a single value is given for all cattle less than 6 months of age.

Noble et al. (26) investigated changes in plasma lipids of lambs during the first 8 days after birth. They observed large increases in the cholesteryl ester, triglyceride, and phospholipid fractions when the lambs were suckled normally but not when they were raised on a diet of reconstituted low fat dried milk powder. The rise in plasma lipids in lambs suckled by ewes was attributed to the high intake of fat provided by the colostrum and the milk.

Pond et al. (27) found that serum cholesterols decreased soon after weaning in both conventional and miniature pigs. The earliest samples in their study were taken at 3-5 days of age, so that they did not observe the low levels found on the day of birth in our experiments

TABLE I

Lipid and Cholesterol Content of Colostrum and Milk from Cows, Ewes, and Sows

Species	Type of milk	No. of samples	Total lipid, g/100 ml	Total cholesterol, mg/100 ml
Cow	Colostrum	10	8.3 ± 0.9[a]	71 ± 11[a]
	Whole milk	14	3.5 ± 0.2	13 ± 1
	Skim milk	5	0.1 ± 0.02	3 ± 0.1
Ewe	Colostrum	3	10.3[b]	88[b]
	Whole milk	6	7.4 ± 0.5	24 ± 3.4
Sow	Colostrum	4	4.0 ± 0.1	93 ± 29
	Whole milk	6	8.2 ± 1.0	42 ± 3

[a]Mean ± SEM.

[b]Ranges (9.5-11.9) and (73-109), respectively.

(Fig. 3A).

From our studies it appeared that the changes in plasma cholesterol levels in young animals were related to diet rather than age. In calves, the plasma cholesterol remained high until they were weaned at 35 days of age (Fig. 1), and in the early and late weaned pigs having no access to pig starter prior to weaning, the decrease in plasma cholesterol occurred just after weaning in each case (Fig. 3B). On the other hand, in lambs (Fig. 2), and in pigs which had access to pig starter from 7 days of age (Fig. 3A), the plasma cholesterols began to drop before the animals were weaned. In these cases, the process of weaning probably began before the animals actually were separated from their mothers. Although the calves had access to calf starter and hay from 10 days of age, they probably consumed very little of these until they were weaned from milk. Unlike the lambs and pigs, which were suckled by their mothers, the calves were on a pail feeding program after 7 days of age and, thus, were assured of an adequate milk supply. Furthermore, calves grow more slowly than lambs or pigs at this stage and, therefore, have relatively less need to supplement their diet with solid feed to meet increasing nutrient requirements.

The results of the experiment in which skim milk was fed indicated that milk lipid was an important factor in the development of hypercholesterolemia in suckling calves (Fig. 1B). This agrees with conclusions reached by earlier workers in studies with calves (7,10), lambs (26), rabbits (12), and rats (14). The lipid content of milk also appears to be an important factor in the postnatal increase of serum cholesterol levels in humans (4,5,28-30). It seems probable that the high cholesterol and lipid content of colostrum (Table I) accounts for the rapid elevation of plasma cholesterol in calves, lambs, and pigs during the first few days of life.

Our analyses of the total lipid and cholesterol content of colostrum and milk from cows, ewes, and sows (Table I) gave results that agree reasonably well with values reported in other literature (31-38). In general the levels of cholesterol and total lipid are considerably higher in colostrum than in milk. The one exception to this was sow's milk, which was found to have a higher total lipid content than colostrum, both in our experiments and in reports from other laboratories (32,37). Deuel (35) quotes the relatively high value of 145 mg/100 ml for cholesterol in sow's milk; but our analyses, carried out on the same samples used for determination of total lipids, gave an average value of only 42 mg/100 ml compared to 93 mg/100 ml for colostrum (Table I).

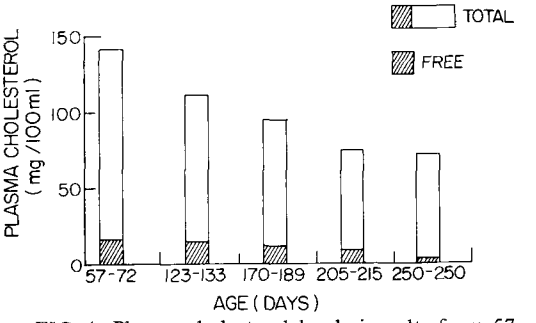

FIG. 4. Plasma cholesterol levels in colts from 57 to 250 days of age. Each bar represents the mean cholesterol level in 2-4 colts.

Our studies with colts were less complete than those with the other three species, and no samples were obtained before 2 months of age. From then until 7-8 months of age, there was a gradual decrease in the level of plasma cholesterol (Fig. 4). There was no apparent change in the downward trend that might be correlated with weaning; but the weaning process may have been gradual, since the colts had access to the adults' feed and since milk production by mares commonly falls off significantly after ca. 8 weeks of lactation. Fonnesbeck and Symons (39) found plasma cholesterol levels of 57-87 mg/100 ml in adult Standardbred geldings, depending upon the type of forage or forage-grain diet fed.

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REFERENCES

1. Sperry, W.M., Amer. J. Dis. Child. 51:84 (1936).
2. Deuel, H.J., Jr., "The Lipids," Vol. II, Interscience Publishers, Inc., New York, 1955, p. 397.
3. Robertson, E.C., in "Nutrition," Vol. III, Edited by G.H. Beaton and E.W. McHenry, Academic Press, Inc., New York, 1966, p. 56.
4. Pomeranze, J., A. Goalwin and L.B. Slobody, Amer. J. Dis. Child. 95:622 (1958).
5. Sweeney, M.J., J.N. Etteldorf, W.T. Dobbins, B. Somervill, B. Fischer and C. Ferrell, Pediatrics 27:765 (1961).
6. Hahn, P., and O. Koldovsky, "Utilization of Nutrients during Postnatal Development," Pergamon Press, New York, 1966, p. 98.
7. Shope, R.E., J. Biol. Chem. 80:141 (1929).
8. Zaletel, J.H., R.S. Allen and N.L. Jacobson, J. Dairy Sci. 35:1046 (1952).
9. Jacobson, N.L., J.H. Zaletel and R.S. Allen, Ibid. 36:832 (1953).
10. Shannon, A.D., and A.K. Lascelles, Aust. J. Biol. Sci. 19:831 (1966).
11. Bragdon, J.H., Circulation 5:641 (1952).
12. Friedman, M., and S.O. Byers, Amer. J. Physiol. 201:611 (1961).

13. Carroll, K.K., Can. J. Biochem. 42:79 (1964).
14. Harris, R.A., J.E. MacNintch and F.W. Quackenbush, J. Nutr. 90:40 (1966).
15. Laird, C.W., and R.R. Fox, Life Sci. 9, Part II:1243 (1970).
16. Hodges, R.G., W.M. Sperry and D.H. Andersen, Amer. J. Dis. Child. 65:858 (1943).
17. Weise, H.F., M.J. Bennett, I.H.G. Braun, W. Yamanaka and E. Coon, Amer. J. Clin. Nutr. 18:155 (1966).
18. Lopez-S, A., W.A. Krehl and R.E. Hodges, Ibid. 20:808 (1967).
19. Wem, E.E., and E.B. Wilcox, J. Amer. Diet. Ass. 61:155 (1972).
20. Whyte, H.M., and I.L. Yee, Australas. Ann. Med. 7:336 (1958).
21. Hamilton, R.M.G., K.K. Carroll and G.K. Macleod, Proceedings of World Congress (International Society for Fat Research and American Oil Chemists' Society), Chicago, 1970, Abst. No. 166.
22. Sperry, W.M., and M. Webb, J. Biol. Chem. 187:97 (1950).
23. Association of Official Agricultural Chemists, 10th Edition, Section 15.029 (1965).
24. Wiggers, K.D., N.L. Jacobson and R. Getty, Atherosclerosis 14:379 (1971).
25. Tumbleson, M.E., and D.P. Hutcheson, Proc. Soc. Exper. Biol. Med. 138:1083 (1971).
26. Noble, R.C., W. Steele and J.H. Moore, Lipids 6:26 (1971).
27. Pond, W.G., R.J. Banis, L.D. Van Vleck, E.F. Walker, Jr. and P. Chapman, Proc. Soc. Exper. Biol. Med. 127:895 (1968).
28. Fomon, S.J., and D.J. Bartels, Amer. J. Dis. Child. 99:27 (1960).
29. Goalwin, A., and J. Pomeranze, Arch. Pediat. 79:58 (1962).
30. Fomon, S.J., Bull. N.Y. Acad. Med. 47:569 (1971).
31. Shope, R.E., and J.W. Gowen, J. Exp. Med. 48:21 (1928).
32. Braude, R., M.E. Coates, K.M. Henry, S.K. Kon, S.J. Rowland, S.Y. Thompson and D.M. Walker, Brit. J. Nutr. 1:64 (1947).
33. Nataf, B., O. Mickelsen, A. Keys and W.E. Petersen, J. Nutr. 36:495 (1948).
34. Barnicoat, C.R., A.G. Logan and A.I. Grant, J. Agr. Sci. 39:237 (1949).
35. Deuel, H.J., Jr., "The Lipids," Vol. II, Interscience Publishers, Inc., New York, 1955, p. 794.
36. Ling, E.R., S.K. Kon and J.W.G. Porter, in "Milk: The Mammary Gland and its Secretions," Vol. II, Edited by S.K. Kon and A.T. Cowie, Academic Press, Inc., New York, 1961, p. 231.
37. Bowland, J.P., in "Swine in Biomedical Research," Edited by L.K. Bustad, R.O. McClellan and M.P. Burns, Battelle Memorial Institute, Pacific Northwest Laboratory, Richland, Washington, 1966, p. 97.
38. Noble, R.C., W. Steele and J.H. Moore, J. Dairy Res. 37:297 (1970).
39. Fonnesbeck, P.V., and L.D. Symons, J. Animal Sci. 28:216 (1969).

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Changes in Liver Lipid Composition of Male Rats Fed Rapeseed Oil Diets[1,2]

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ABSTRACT

Studies are reported on the effect of feeding diets containing rapeseed oils differing in their erucic acid content to male weanling rats for 16 weeks. Rapeseed oil high in erucic acid depressed growth. Total lipids, lipid phosphorous and cholesterol, in the livers were not significantly different between the experimental groups. The fatty acid composition of the total liver lipids, the neutral lipids, phosphatidylethanolamine and phosphatidylcholine are documented. Erucic and eicosenoic acids were found in all lipid classes at the same relative concentration; the amount being incorporated was proportional to that found in the dietary oil. The positional analysis of phosphatidylethanolamine and phosphatidylcholine are presented. Erucic acid was incorporated preferentially at position two of these phospholipids, whereas, twice the level of eicosenoic acid was found at position one, compared to that which occurred at the two position.

INTRODUCTION

Several investigations have been reported on the effect of dietary rapeseed oils on the fatty acid composition of different tissues of the rat (1-10). The comparative effect of rapeseed oils containing high or low levels of erucic (*cis*-13-docosenoic) acid (22:1) was studied (5,7-10). Although data are available for total composition of tissue lipids within the first week of feeding rapeseed oils (6-9) and after long term feeding experiments (10), little is known about the fatty acid composition of particular lipid classes (11,12).

It has been established that feeding rapeseed oil, high in 22:1 concentration, causes severe fat accumulation in heart, adrenal, and skeletal muscle within the first week of feeding. This fatty infiltration gradually disappears after rats are kept several months on these diets (13). The early cardiac fat accumulation is due mainly to an increase in the concentration of triglycerides and free fatty acids, while the phospholipid and cholesterol concentrations remain constant (7, 9,14). The concentration of 22:1 was shown to be much lower in the total phospholipids than in the neutral lipids of the heart (1,9), adrenal (2), and liver (1,3,11,12); in fact no 22:1 was detected in the phospholipids of the liver in long term feeding trials.

In this study male weanling rats were fed for 16 weeks various rapeseed oils which differed in their 22:1 concentration. The livers were analyzed for their total fatty acid composition and for the fatty acid composition of several major lipid classes: neutral lipids, phosphatidylethanolamine (PE), and phosphatidylcholine (PC). In addition the positional distribution of fatty acids in PE and PC was determined.

MATERIALS AND METHODS

Male Sprague-Dawley rats, three weeks old, obtained from Bio-Breeding, Ottawa, Ontario, were selected randomly, caged in pairs, and fed ad libitum for 16 weeks. Five groups of four rats were fed a semisynthetic diet containing 20% casein (vitamin free), 30% cornstarch, 20% sucrose, 1% vitamin mixture (15), 4% USP XIV salt mixture (30 ppm zinc), and 5% alfa floc. Diet one was supplemented with 5% lard and an additional 15% cornstarch; diet two with 20% corn oil; diet three with 20% *Brassica napus* var. Oro (Oro) processed by Cooperative Vegetable Oil Ltd., Altona, Manitoba; diet four with 20% *B. campestris* var. Span (Span) processed by Western Canada Processors, Lethbridge, Alberta; diet five with 20% rapeseed oil, which is obtained from a seed mixture of *B. campestris* var. Echo (15%) and Arlo (85%) (processed by the latter plant). Hereafter, this mixture will be referred to as RSO. After 16 weeks on the diets, the rats were killed by stunning followed by exsanguination. The livers were removed immediately; washed in ice-cold saline, and homogenized in distilled water using a Potter-Elvehjem homogenizer. Total lipids were extracted twice, according to the procedure of Bligh and Dyer (16).

Lipids were transesterified by refluxing with

[1]This article represents part of an extensive experiment carried out by Agriculture Canada to investigate the nutritional value of rapeseed oils (see ref. 15).

[2]Contribution No. 497 from the Animal Research Institute.

TABLE I

Fatty Acid Composition of Dietary Oils[a]

Fatty[b] acid	Mole %				
	Lard	Corn	Oro	Span	RSO
14:0	2.0	trace	0.1	0.1	0.1
16:0	28.4	10.9	5.0	4.0	4.0
16:1	2.8	0.1	0.3	0.3	0.3
18:0	18.0	1.7	2.5	1.7	1.7
18:1	38.1	24.3	62.2	55.9	36.2
18:2	7.9	61.1	18.9	20.0	15.1
18:3	0.4	0.9	6.7	8.3	5.9
20:1	0.8	0.2	1.9	3.9	12.3
20:2	0.4	trace	0.1	0.5	0.5
22:0	—[c]	0.1	0.3	0.4	0.4
22:1	—	—	1.6	4.3	22.3
22:2	—	—	trace	0.3	0.1
24:0	—	0.2	0.3	0.3	1.0
24:1	—	—	—	—	trace

[a]Oro = *Brassica napus* var. Oro; Span = *B. campestris* var. Span; RSO = mixture of *B. campestris* var. Echo and Arlo.

[b]Number of carbon atoms; number of double bonds.

[c]No detectable amounts of fatty acids were observed.

5% (w/w) dry HCl gas in anhydrous methanol for 45 min (17), and the methyl esters purified by thin layer chromatography (TLC). Lipid classes were separated by TLC by means of a two step development procedure (18) to obtain neutral lipids, PE and PC. Bands were visualized under UV light after the chromatograms were sprayed with Rhodamine B.

PE and PC were hydrolyzed enzymatically using snake venom from king cobra (*Ophiophagus hannah*) (Sigma Chemical Co., St. Louis, Mo.) according to Weber et al. (19). The products resulting from phospholipase A hydrolysis were isolated by TLC using $CHCl_3$:CH_3OH:H_2O (65:25:4) as developing solvent.

Free acid methyl esters were analyzed using a gas liquid chromatograph (GLC) (Packard Model 420) equipped with a flame ionization detector. A 200 by 4 mm glass column packed with 5% butanediol succinate on 80/100 mesh Chromosorb G (high performance) was used isothermally at 200 C. Nitrogen was the carrier gas at a rate of 60 ml/min. Peaks were identified by comparison with authentic standards (Nu Chek Prep, Elysian, Minn.) and by the equivalent chain length technique, quantitated by use of a digital integrator (Infotronic 208E), and expressed as mole per cent. Analysis of variance was performed on all data, and significant differences at the 1% level ($P < 0.01$) were determined using Duncan's Multiple Range test. Free and esterified cholesterol (20) and total phosphorous (21) were determined by standard procedures.

RESULTS

The fatty acid composition of the dietary oils is given in Table I. Characteristic low levels of saturated fatty acids were found in all oils; corn oil, however, contained slightly higher levels of palmitic acid (16:0). A high concentration of monoenoic acids, oleic (18:1), eicosenoic (20:1), and erucic (22:1), was common to all rapeseed oils. RSO, Span, and Oro contained decreasing amounts of 20:1 and 22:1, due mostly to a substitution of 18:1. Linoleic acid (18:2) was the major fatty acid in corn oil (57.9%), and it occurred at 15-20% in the rapeseed oils. Linolenic acid (18:3) was found in appreciable amounts (6-8%) in all the rapeseed oils.

Body wt gains of male rats fed diets containing RSO or lard were lower compared to rats fed corn, Oro, or Span oil diets (Table II). Previously it has been reported from this laboratory that a significant increase in hepatic fat occurred in rats fed RSO or Span for two weeks (15). The total lipid, lipid phosphorous, and cholesterol content/g of liver was not significantly different between the treatments

TABLE II

Weight Gain, Liver Weights, Total Liver Lipids, Cholesterol and Phosphorous[a]

Diet	Weight gain g	Liver wet weight g	Total lipids mg/g liver	Total phosphorous mg/g liver	Total cholesterol mg/g liver
Lard	406.7 ± 11.6[b]	15.4 ± 0.7	31.6 ± 3.1	0.60 ± 0.04	1.5 ± 0.3
Corn	443.3 ± 18.1	12.7 ± 1.0	51.0 ± 5.5	0.78 ± 0.05	2.6 ± 0.7
Oro	434.5 ± 18.2	12.1 ± 1.2	37.4 ± 2.2	0.73 ± 0.02	1.8 ± 0.2
Span	433.9 ± 11.7	15.3 ± 1.0	41.6 ± 4.3	0.65 ± 0.05	1.9 ± 0.2
RSO	406.5 ± 6.9	14.3 ± 0.7	38.2 ± 4.9	0.75 ± 0.03	1.4 ± 0.1

[a]Oro = *Brassica napus* var. Oro; Span = *B. campestris* var. Span; RSO = mixture of *B. campestris* var. Echo and Arlo.

[b]Mean ± standard error of the mean of four rats.

TABLE III

Fatty Acid Composition of Total Liver Lipids from Male Rats 16 Weeks on Experimental Diets[a]

Fatty[b] acid	Diet					
	Lard	Corn	Oro	Span	RSO	SEM
14:0	1.1[1f]	0.4[2]	0.3[2]	0.5[2]	0.8[1,2]	0.1
16:0	29.5[1]	25.3[1,2]	19.4[2,3]	15.4[3]	19.3[2,3]	1.3
16:1	7.9	1.0[1]	1.7[1]	2.4[1]	3.2[1]	0.6
18:0	13.2[1,2]	10.6[1,2]	16.3[1]	7.7[2]	11.2[1,2]	1.8
18:1	31.7[2]	16.8	34.2[2]	44.5[1]	35.6[1,2]	2.1
18:2	5.8	31.5	13.9[1]	14.2[1]	11.7[1]	0.8
18:3	trace[2]	0.3[2]	1.6[1]	2.3[1]	1.8[1]	0.2
20:1	0.4[2]	0.6[2]	1.1[1,2]	1.8[1]	3.5	0.2
20:2	0.7[1,2]	0.9[1]	0.3[2]	0.5[2]	0.3[2]	0.08
20:4	6.7[1]	8.4[1]	7.5[1]	7.2[1]	7.2[1]	0.7
22:1	—[g]	—	0.1[1]	0.4[1]	1.7	0.05
22:6	0.8[2]	0.7[2]	1.4[1,2]	0.9[1,2]	1.7[1]	0.2
24:0	0.4[1,2]	0.8[1]	trace[2]	0.1[2]	trace[2]	0.1
Minor S[c]	1.1	1.8	1.3	1.3	1.3	
Minor P[d]	0.7	1.0	0.9	0.7	0.8	
% S	45.3	38.9	37.3	25.0	32.6	
% M	40.0	18.4	37.1	49.1	44.0	
% P	14.7	42.8	25.6	25.8	23.5	
S/U[e]	0.83	0.64	0.59	0.33	0.48	

[a]Oro = *Brassica napus* var. Oro; Span = *B. campestris* var. Span; RSO = mixture of *B. campestris* var. Echo and Arlo.

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

[c]Mole % of all trace amounts of saturated fatty acids: 15:0, 17:0 and 20:0.

[d]Mole % of all trace amounts of polyunsaturated fatty acids: 20:3, 20:5, 22:4 and 22:5.

[e]Ratio of total molar % saturated fatty acids to total molar % unsaturated fatty acids.

[f]Analysis of variance was calculated for each set of methyl esters. The mean of four rats on each diet and the pooled standard error of the mean (SEM) are given. Identical superscript numbers indicate no significant difference at the 1% level using Duncan's Multiple Range test.

[g]No detectable amounts of fatty acid were observed.

after the rats were maintained on these diets for 16 weeks (Table II).

The total fatty acid composition of the liver lipids of male rats fed the experimental diets for 16 weeks are presented in Table III. The composition of neutral lipids, PE and PC, isolated from the total hepatic lipids by TLC, are presented in Tables IV, V, and VI, respectively.

Erucic acid was incorporated into the liver lipids of rats maintained on diets containing rapeseed oils: Oro at 0.1%, Span at 0.4%, and RSO at 1.7%. Surprisingly the concentration of 22:1 was found to be the same in both the neutral lipids and phospholipids (PE and PC), contrary to earlier reports that 22:1 was found only in the neutral lipid fraction of the liver lipids (1,3,12). The concentration of 22:1 in the liver lipids was proportional to its concentration in the dietary rapeseed oils but at a much lower concentration.

Although eicosenoic acid was found in the liver lipids of the control groups (lard 0.2-0.4%, corn 0.4-0.6%), much higher concentrations were present in rats fed the rapeseed oils. The concentration of 20:1 appeared to be related to the dietary intake, although the contribution from β-oxidation of 22:1 and chain elongation of 18:1 cannot be eliminated. In rats fed RSO, for example, the 20:1 content in all lipid classes (3.3-3.5%) was greater than the 22:1 content (1.1-1.7%), even though the composition of the dietary RSO showed the opposite relationship (12.3%, 20:1; 22.3%, 22:1). The concentration of 20:1 was identical in the neutral lipids and phospholipids of the liver.

Oleic acid was the major dietary fatty acid in all experimental groups, except those fed corn oil; a corresponding increase in the concentration of 18:1 in all lipid class compositions was observed, in particular that of the neutral lipids. The concentration of 18:1 in the neutral lipid

TABLE IV

Fatty Acid Composition of Neutral Lipids from Male Rat Livers 16 Weeks on Experimental Diets[a]

Fatty acid	Diet					
	Lard	Corn	Oro	Span	RSO	SEM
14:0	1.6	0.5[1]	0.8[1]	0.7[1]	1.0[1]	0.1
16:0	35.2	24.6[1]	19.5[1,2]	13.3[2]	18.5[1,2]	1.4
16:1	9.1	1.0[2]	2.6[1,2]	3.1[1]	3.8[1]	0.4
18:0	2.7[1]	1.9[1]	2.8[1]	1.4[1]	1.6[1]	0.5
18:1	46.0	23.5	56.0[1]	57.8[1]	54.5[1]	1.0
18:2	3.9	42.6	14.3[1]	16.4[1]	11.4	0.5
18:3	trace[2]	0.2[2]	1.5[1]	2.9	2.0[1]	0.2
20:1	0.3[2]	0.4[2]	0.9[1,2]	1.5[1]	3.5	0.2
20:2	0.1[1]	0.5	0.1[1]	0.2[1]	0.2[1]	0.05
20:4	0.4[1]	2.5	0.6[1]	1.0[1]	0.6[1]	0.1
22:1	—	—	0.1[1]	0.3[1]	1.7	0.07
22:6	—	trace[1]	0.1[1]	0.1[1]	trace[1]	0.03
24:0	trace[1]	0.3	—	trace[1]	—	0.02
Minor S	0.7	1.1	0.7	1.1	0.9	
Minor P	—	0.8	0.3	0.1	0.2	
% S	40.2	28.4	23.8	16.5	22.0	
% M	55.2	24.9	59.6	62.7	63.5	
% P	4.4	46.6	16.9	20.7	14.4	
S/U	0.67	0.40	0.31	0.20	0.28	

[a]See Table III Footnotes.

fraction of rats fed lard was 46%, whereas rats maintained on diets containing Oro, Span, or RSO had higher levels of 18:1 which were remarkably similar to each other (Table IV). The latter observation reflected the similarity of the monoenoic acid composition of Oro, Span, and RSO, and was, no doubt, a consequence of rapid β-oxidation of 22:1 and 20:1 to 18:1. The 18:1 level of PC was slightly higher in the groups fed rapeseed oils than in the control groups, whereas in PE the level of 18:1 was increased significantly ($P < 0.01$). A similarity in the level of 18:1 was observed between the three rapeseed oil fed groups,

TABLE V

Fatty Acid Composition of Phosphatidylethanolamine from Male Rat Livers 16 Weeks on Experimental Diets[a]

Fatty acid	Diet					
	Lard	Corn	Oro	Span	RSO	SEM
14:0	0.1[1]	0.1[1]	0.2[1]	0.2[1]	0.3	0.03
16:0	24.7[1]	21.0[1,2]	16.8[2,3]	12.8[3]	17.7[1,2,3]	1.7
16:1	1.5[1]	0.5[2]	0.5[2]	0.8[1,2]	0.8[1,2]	0.2
18:0	32.9[1]	26.2[1]	28.0[1]	23.9[1]	22.8[1]	2.6
18:1	11.1[2]	8.7[2]	16.0[1]	20.0	16.1[1]	0.9
18:2	4.1	14.0[1]	13.7[1]	11.7[1]	9.5[1]	1.0
18:3	—	—	0.3[2]	1.2[1]	0.9[1,2]	0.2
20:1	0.3[1]	0.5[1]	1.0[1]	2.0	3.3	0.2
20:2	0.6[1]	1.2[1]	0.4[1]	0.9[1]	0.8[1]	0.3
20:4	16.9[1]	20.0[1]	15.4[1]	17.7[1]	17.5[1]	2.2
22:1	—	—	0.3[1]	0.5[1]	1.1	0.1
22:6	4.1[1]	2.5[1]	4.7[1]	5.0[1]	6.9[1]	1.1
24:0	1.3[1]	2.5[1]	—	—	—	0.4
Minor S	1.2	1.3	1.3	1.6	1.1	
Minor P	1.2	1.4	1.6	1.6	1.3	
% S	60.2	51.1	46.3	38.5	41.9	
% M	12.9	9.7	17.8	23.3	21.3	
% P	26.9	39.1	36.1	38.1	36.9	
S/U	1.51	1.05	0.86	0.63	0.72	

[a]See Table III Footnotes.

TABLE VI

Fatty Acid Composition of Phosphatidylcholine from Male Rat Livers 16 Weeks on Experimental Diets[a]

Fatty acid	Diet					
	Lard	Corn	Oro	Span	RSO	SEM
14:0	0.3[1]	0.3[1]	0.2[1]	0.2[1]	0.2[1]	0.03
16:0	25.3[1,2]	30.7[1]	21.0[2]	20.2[2]	21.0[2]	1.6
16:1	1.8	0.8[1]	0.2	0.9[1]	0.8[1]	0.1
18:0	33.7[1]	26.1[1]	25.5[1]	24.1[1]	22.6[1]	2.5
18:1	11.5[2]	6.6	13.6[1,2]	16.5[1]	13.4[1,2]	0.7
18:2	6.2[2]	11.6[1]	13.0[1]	9.7[1,2]	9.9[1,2]	0.9
18:3	—	trace	0.6[1]	0.5[1]	0.6[1]	0.2
20:1	0.2[1]	0.5[1]	1.1	2.1	3.5	0.1
20:2	1.4[1]	1.2[1]	0.3	0.7[2]	0.8[2]	0.1
20:4	15.9[1]	18.6[1]	19.3[1]	19.9[1]	20.9[1]	2.6
22:1	—	—	0.2	0.5	1.1	0.05
22:6	1.5[1]	0.9[1]	2.5[1]	2.5[1]	3.3[1]	0.6
24:0	0.7[1]	0.8[1]	0.1[2]	trace[2]	—	0.1
Minor S	0.6	1.3	1.0	1.5	0.9	
Minor P	0.9	0.6	1.6	0.8	1.0	
% S	60.6	59.2	47.8	46.0	44.7	
% M	13.5	7.9	15.1	20.0	18.8	
% P	25.9	32.9	37.3	34.1	36.5	
S/U	1.54	1.45	0.91	0.85	0.81	

[a]See Table III Footnotes.

although the Span group appeared to contain a slightly higher level than either the Oro or RSO fed groups.

The relative concentration of 16:0 was influenced significantly by the different diets, while the concentration of 18:0 was not affected. A marked decrease in the concentration of 16:0 was observed in all lipid classes of all groups fed rapeseed oil compared to the groups fed corn oil or lard. The decrease was most pronounced with the group maintained on Span oil. The level of 18:0 was not significantly different between the experimental groups in either of the neutral lipids, PE or PC.

The concentration of 18:2 in the neutral lipids was related directly to its concentration in the diet. The neutral lipids of the group fed a diet containing lard had the lowest level of 18:2; the group fed the corn oil containing diet had the highest level, whereas the group fed the rapeseed oil(s) containing diets had intermediate levels of 18:2 in the neutral lipids. The influence of dietary 18:2 on the phospholipid composition was much less dramatic. The concentration of 18:2 was significantly lower only in the lard group; differences between the concentration of 18:2 in corn and rapeseed oils did not give rise to significantly different levels of incorporation into the phospholipids (Tables V and VI).

Low levels of 18:3 were found in all lipid classes of rats fed diets containing rapeseed oils. Linolenic acid was incorporated into the neutral lipids (1.5-2.9%) and somewhat less into the phospholipids (0.3-1.2%) of the liver.

The relative proportion of 20:4 in the total lipids of rat livers was not statistically different between the experimental groups. The same pattern was observed in the fatty acid profile of PE and PC isolated from the total liver lipids. On the other hand, the relative concentration of 22:6 appeared to be greater in the phospholipids of rats fed rapeseed oils compared to the control groups. Characteristic differences between PE and PC were maintained; 22:6 was more abundant in PE than in PC.

The ratio of total saturated to total unsaturated fatty acids are included in Tables III to VI. Lower ratios were observed consistently in all liver lipid classes of rats fed diets containing Oro, Span, or RSO, compared to rats fed diets containing lard or corn oil. The ratios in the phospholipids (PE and PC) decreased progressively as the erucic acid content of the dietary rapeseed oils increased. The lower ratios were due to a combination of a lower concentration of saturated and a greater abundance of monounsaturated fatty acids.

The positional distribution of fatty acids esterified to position one and two of PE and PC are presented in Tables VII and VIII, respectively. The characteristic pattern of phospholipids was retained; saturated acids predominated in position one, whereas polyunsaturated

TABLE VII

Distribution of Fatty Acids Esterified to the 1- and 2- Position of Phosphatidylethanolamine from Male Rat Livers 16 Weeks on Experimental Diets[a]

Fatty acid	Diet											
	Lard		Corn		Oro		Span		RSO		SEM	
	1	2	1	2	1	2	1	2	1	2	1	2
14:0	0.6	0.2	0.3	0.3	0.6	0.1	0.2	0.1	0.3	0.2	0.2	0.07
16:0	33.4	14.9	31.8	12.7	25.8	10.7	23.9	6.9	28.4	11.0	1.5	1.4
16:1	1.0	1.3	0.3	0.5	0.4	0.4	—	0.3	0.4	0.6	0.3	0.1
18:0	49.3	21.5	49.1	20.1	54.0	22.8	50.3	15.9	45.5	17.3	2.1	2.7
18:1	11.2	10.7	10.8	7.1	13.2	13.2	17.2	13.5	14.0	12.2	1.5	1.0
18:2	0.3	4.5	1.3	11.3	0.4	7.3	1.0	7.5	1.0	7.0	0.2	1.2
18:3	0.1	—	0.1	trace	—	0.2	trace	0.6	—	0.5	0.03	0.1
20:1	0.5	0.3	0.9	0.4	1.4	1.4	2.5	1.5	5.5	2.9	0.3	0.2
20:2	0.1	1.2	1.6	1.1	0.2	0.5	0.5	0.5	0.5	0.5	0.07	0.08
20:4	0.5	30.6	0.3	32.5	0.3	26.6	0.4	34.9	0.7	28.4	0.1	2.3
22:1	—	—	—	—	—	0.4	—	0.9	0.5	1.6	0.04	0.1
22:6	—	8.7	—	5.2	—	11.7	—	13.8	—	14.6	—	1.7
24:0	—	2.4	0.2	4.1	—	trace	—	0.3	0.1	trace	0.06	0.7
Minor S	3.2	0.8	3.2	2.0	3.5	1.5	4.1	1.0	3.2	1.1		
Minor P	—	2.6	—	2.8	—	3.1	—	2.0	—	2.2		
% S	86.5	39.8	84.6	39.2	83.9	35.1	78.5	24.2	77.5	29.6		
% M	12.7	12.3	12.0	8.0	15.0	15.4	19.7	16.2	20.4	17.3		
% P	1.0	47.6	3.3	52.9	0.9	49.4	1.9	59.3	2.2	53.2		
S/U	6.3	0.66	5.5	0.64	5.3	0.54	3.6	0.32	3.4	0.42		

[a]See Table III Footnotes.

acids predominated in position two. Erucic acid was incorporated preferentially into position two of PE (Oro 0.4%, Span 0.9%, RSO 1.6%) and PC (Oro 0.3%, Span 1.1%, RSO 1.2%). Only trace amounts of 22:1 were found in position one of PE and PC in the groups fed Oro or Span. Ca. one-half as much 22:1 occurred in position two of PE and PC compared to that found in position one of the group fed RSO. Eicosenoic acid, on the other hand, was incorporated preferentially into position one of PE and PC, and occurred in position two at ca. half that concentration. Linolenic acid was incorporated into position one of both phospholipids from rats fed rapeseed oils. Furthermore, the concentration of 22:6 in PE was significantly greater in rats fed rapeseed oils, suggesting a rapid chain elongation and desaturation of 18:3.

The influence of dietary rapeseed oils on the composition of the one and two position in PE was similar; the concentration of saturated fatty acids was lower, of monounsaturated acids was higher, and of polyunsaturated acids not significantly different ($P < 0.01$) from those of the control groups. The greatest changes occurred in rats fed Span and RSO. A similar pattern was observed in the composition of position one in PC. However, the composition of position two in PC was influenced less by the dietary rapeseed oils; the concentration of saturates and mono- and polyunsaturates were between those of the control groups fed corn oil or lard.

The ratio of saturated to unsaturated fatty acids in each position of each diet is given in Tables VII and VIII for PE and PC, respectively. Slightly lower ratios were observed in rats fed rapeseed oils as compared to those fed corn oil or lard. A significant ($P < 0.01$) change in ratio was observed for position one and two of PE and position one of PC, whereas, the ratio in position two of PC remained remarkably similar regardless of the diet fed.

DISCUSSION

Previous investigations have indicated cardiac fat accumulation within the first week of feeding rapeseed oils high in 22:1 to male rats (6-9, 11,13-15) and necrotic heart lesions when these oils were fed for periods of several months (5,7,9,11,13,15,22). Erucic acid has been implicated as causing the lesions (23). Three varieties of rapeseed oils, which differed in their 22:1 content, were studied to determine the extent of incorporation of this acid into the different hepatic lipid classes.

It can be concluded from this study that 22:1 and 20:1 were incorporated ca. equally into hepatic neutral lipids and phospholipids when rapeseed oils containing these acids were fed. This contrasts earlier reports which indi-

TABLE VIII

Distribution of Fatty Acids Esterified to the 1- and 2- Positions of Phosphatidylcholine from Male Rat Livers 16 Weeks on Experimental Diets[a]

Fatty acid	Diet											
	Lard		Corn		Oro		Span		RSO		SEM	
	1	2	1	2	1	2	1	2	1	2	1	2
14:0	0.4	0.2	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.3	0.04	0.1
16:0	37.0	12.1	36.3	15.9	27.6	11.3	28.3	10.6	28.3	13.3	1.4	1.5
16:1	1.6	2.3	0.6	0.3	0.5	0.5	0.7	0.5	0.5	0.6	0.2	0.2
18:0	44.9	13.1	46.0	15.5	48.3	16.5	47.2	14.5	44.4	15.3	2.9	2.1
18:1	12.0	15.1	7.9	5.4	14.3	13.6	14.1	13.6	12.4	11.1	1.4	1.1
18:2	1.3	11.1	2.2	13.7	1.9	15.7	1.9	13.4	1.8	13.5	0.3	1.1
18:3	—	—	—	—	—	0.3	—	0.4	—	0.2	—	0.1
20:1	0.6	0.3	1.3	0.4	2.9	1.0	3.0	1.2	7.2	2.2	0.6	0.3
20:2	0.2	3.0	2.3	1.2	0.7	0.6	0.7	0.6	0.8	0.6	0.1	0.2
20:4	0.3	31.7	0.3	39.6	0.5	29.7	0.6	34.9	0.8	31.8	0.1	2.9
22:1	—	—	—	—	0.1	0.3	0.1	1.1	0.6	1.2	0.03	0.2
22:6	—	4.8	—	2.7	—	5.8	—	5.9	—	5.6	—	0.6
24:0	—	1.8	—	2.5	—	0.1	—	0.1	—	1.2	—	0.6
Minor S	1.6	0.9	2.8	1.1	2.8	1.2	3.2	1.3	3.2	1.0		
Minor P	—	3.7	—	1.5	—	3.4	—	1.7	—	2.0		
%S	83.9	28.1	85.3	35.1	78.9	29.2	78.9	26.6	76.1	31.1		
%M	14.2	17.7	9.8	6.1	17.8	15.4	17.9	16.4	20.7	15.1		
%P	1.8	54.3	4.8	58.7	3.1	55.5	3.2	56.9	3.4	53.7		
S/U	5.2	0.39	5.8	0.54	3.8	0.41	3.7	0.36	3.2	0.45		

[a]See Table III Footnotes.

cated, after long term feeding trials, that 22:1 was found exclusively in the neutral lipids (1,3,12). The concentration of 22:1 in all lipid classes was in the same ratio as the concentration of this acid in the diet, whereas the concentration of 20:1 showed the proportional relationship, provided an amount present in the control groups is subtracted first. The relative concentration of 22:1 was less than that of 20:1 in all lipid classes, even though in Oro and Span oil both acids occurred at the same relative concentration, and in RSO oil the concentration of 22:1 was twice that of 20:1. The level of 18:1 was significantly higher in the hepatic lipids of rats fed rapeseed oils compared to those fed corn oil or lard, suggesting a rapid β-oxidation of 22:1 and 20:1 to 18:1. Oxidation of long chain monoenoic acids has been demonstrated in rats (24-26). Presumably, 20:1 is a metabolic intermediate in the oxidation of 22:1, accounting for its greater abundance in hepatic lipids (25).

Investigations into the positional distribution of 22:1 and 20:1 in PE and PC revealed that 22:1 was incorporated preferentially into position two and 20:1 into position one. Although no attempts were made to characterize the position of the double bonds in 22:1 and 20:1, it is recognized that erucic acid in rapeseed oils is exclusively 13-*cis*-docosenoic acid and the eicosenoic acid in rapeseed oil a mixture of ca. 75% 11-*cis*- and 25% 13-*cis*-eicosenoic acids (27). Little is known regarding the specific distribution of isomeric long chain monoenes (20:1 and 22:1) in phospholipids. According to Brockerhoff and Ackman (28), 13-*cis*-eicosenoic acid accumulates preferentially in position one and 11-*cis*-eicosenoic acid in either position (variable distribution) of phospholipids. However, they did not determine the distribution of docosenoic acids in rat liver phospholipids (28). In this present investigation, when rapeseed oils were fed to rats, it was observed that 20:1 was present in position one at twice the concentration as that in position two. No attempt was made to distinguish between isomeric 20:1 acids and their origin, be it exogenous or endogenous. An inverse distribution was found for 22:1 in the phospholipids; 22:1 was present in position two at greater abundance than in position one. The pathway by which 22:1 and 20:1 were incorporated into the phospholipids is open to speculation. Hill and Lands (29) have shown that 22:1 did not enter PC via the retailoring route, i.e. acylation of 1-acyl-L-glycerol-3-phosphorylcholine, but that it could be incorporated by acylation of 1-acyl-L-glycerol-3-phosphate, while 20:1 could be incorporated into PC using either substrate.

By expressing the fatty acid composition of the hepatic lipids as a ratio of saturated to

unsaturated fatty acids, a significantly lower ratio was observed for rats fed the rapeseed oil diets in all lipid classes. Generally, the liver lipids of rats fed rapeseed oils had lower concentrations of saturated acids and significantly higher concentrations of monounsaturated acids. A survey of the literature revealed that the monoenoic acid content of PC and PE from normal rat livers is within 6-12%. Representative monoenoic acid concentrations of PC calculated from the data in published papers are: 6.2% (30), 6-8% (31), 7.9 and 9.2% (32), 9.9% (33), 7.2 and 9.8% (34), 7.4% (35), 6.3 and 6.9% (36), 12% (37), 6.5% (38), and 8.2% (39); and of PE are: 7.3-9.1% (31), 6.1 and 9% (34), 8.6% (37), and 5% (38). The monoenoic acid content in PC of rats suffering from essential fatty acid deficiency is within 20 to 30%: 24.7% (33), 24.8% (35), 25.3% (36), 17.7% (39), 24.5% (40), and ca. 20% (41). In the present experiment, rats fed diets containing rapeseed oils had a monoenoic acid concentration between 17 and 23% in PC and PE isolated from the liver lipids. It is tempting to correlate the high concentration of monoenoic acids in PC and PE of rats fed rapeseed oils with a condition similar to that of essential fatty acid deficiency.

Reports have appeared recently in the literature that dermal symptoms similar to essential fatty acid deficiency were observed in hypophysectomized immature rats in spite of an abundance of linoleic and arachidonic acids in the diet, even though only small amounts of 20:3 were observed (42).

It appeared that feeding diets, containing 20% by wt rapeseed oils to rats, brought about a condition of fatty acid imbalance which was not related directly to the concentration of 22:1 and 20:1 found in the hepatic lipid classes. However, a monoenoic acid concentration was found in rats fed rapeseed oils which resembled that found in essential fatty acid deficiency.

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REFERENCES

1. Wagner, H., E. Seelig and K. Bernhard, Hoppe-Seyler's Z. Physiol. Chem. 312:104 (1958).
2. Carroll, K.K., Can. J. Biochem. Physiol. 40:1115 (1962).
3. Craig, B.M., C.G. Youngs, J.L. Beare and J.A. Campbell, Ibid 41:43 (1963).
4. Craig, B.M., and J.L. Beare, Can. J. Biochem. 45:1075 (1967).
5. Rocquelin, G., B. Martin and R. Cluzan, Proceedings of the International Conference on the Science, Technology and Marketing of Rapeseed and Rapeseed Products, Ste. Adèle, Quebec, 1970, p. 405.
6. Beare-Rogers, J.L., Ibid. p. 450.
7. Beare-Rogers, J.L., E.A. Nera and H.A. Heggtveit, Can. Inst. Food Technol. J. 4:120 (1971).
8. Beare-Rogers, J.L., E.A. Nera and B.M. Craig, Lipids 7:46 (1972).
9. Beare-Rogers, J.L., E.A. Nera and B.M. Craig, Ibid. 7:548 (1972).
10. Walker, B.L., Nutr. Metabol. 14:8 (1972).
11. Thoron, A., Ann. Nutr. Aliment. 23:103 (1969).
12. Quan, P.-C, and E. Le Breton, C.R. Acad. Sc. Paris 276D:1585 (1973).
13. Abdellatif, A.M.M., and R.O. Vles, Nutr. Metabol. 12:285 (1970).
14. Houtsmuller, U.M.T., C.B. Struijk and A. Van der Beek, Biochim. Biophys. Acta 218:564 (1970).
15. Kramer, J.K.G., S. Mahadevan, J.R. Hunt, F.D. Sauer, A.H. Corner and K.M. Charlton, J. Nutr. 103:1696 (1973).
16. Bligh, E.G., and W.J. Dyer, Can. J. Biochem. Physiol. 37:911 (1959).
17. Stoffel, W., F. Chu and E.H. Ahrens, Anal. Chem. 31:307 (1959).
18. Neskovic, N.M., and D.M. Kostic, J. Chromatog. 35:297 (1968).
19. Weber, E.J., I.A. de la Roche and D.E. Alexander, Lipids 6:525 (1971).
20. Sobel, C., and A. Fernandez, Clin. Chem. 12:739 (1966).
21. Allen, R.J.L., Biochem. J. 34:858 (1940).
22. Roine, P., E. Uksila, H. Teir and J. Rapola, Z. Ernaehrungswiss. 1:118 (1961).
23. Abdellatif, A.M.M., Nutr. Reviews 30:2 (1972).
24. Carroll, K.K., Can. J. Biochem. Physiol. 40:1229 (1962).
25. Craig, B.M., and J.L. Beare, Can. J. Biochem. 45:1075 (1967).
26. Lemarchal, P., P. Clouet and J.-P. Blond, C.R. Acad. Sc. Paris 274D:1961 (1972).
27. Haeffner, E.W., Lipids 5:430 (1970).
28. Brockerhoff, H., and R.G. Ackman, J. Lipid Res. 8:661 (1967).
29. Hill, E.E., and W.E.M. Lands, Biochim. Biophys. Acta 152:645 (1968).
30. Menzel, D.B., and H.S. Olcott, Ibid. 84:133 (1964).
31. Bear, J.L., and M. Kates, Can. J. Biochem. 42:1477 (1964).
32. Van Den Bosch, H., and L.L.M. Van Deenen, Biochim. Biophys. Acta 106:326 (1965).
33. Van Golde, L.M.G., and L.L.M. Van Deenen, Ibid. 125:496 (1966).
34. Lyman, R.L., J. Tinoco, P. Bouchard, G. Sheehan, R. Ostwald and P. Miljanich, Ibid. 137:107 (1967).
35. Van Golde, L.M.G., W.A. Pieterson and L.L.M. Van Deenen, Ibid. 152:84 (1968).
36. Stancliff, R.C., M.A. Williams, K. Utsumi and L. Packer, Arch. Biochem. Biophys. 131:629 (1969).
37. Wood, R., and R.D. Harlow, Ibid. 131:495 (1969).
38. Fex, G., Biochim. Biophys. Acta 231:161 (1971).
39. Alling, C., A. Bruce, I. Karlsson, O. Sapia and L. Svennerholm, J. Nutr. 102:773 (1972).
40. Johnson, R.R., P. Bouchard, J. Tinoco and R.L. Lyman, Biochem. J. 105:343 (1967).
41. Pudelkewicz, C., and R.T. Holman, Biochim. Biophys. Acta 152:340 (1968).
42. Haeffner, E.W., and O.S. Privett, J. Nutr. 103:74 (1973).

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SHORT COMMUNICATIONS

Preferential Labeling of Brain Cholesterol by {3-^{14}C}D(-)-3-Hydroxybutyrate

ABSTRACT

{3-^{14}C}D(-)-3-hydroxybutyrate or {2-^{14}C}glucose was injected subcutaneously into 15-day old suckling rats. The animals were killed 3, 6, and 24 hr later by decapitation. Brain proteins, cholesterol, glycolipids, and phospholipids were extracted and prepared for counting. The {3-^{14}C}D(-)-3-hydroxybutyrate injected animals showed ca. twofold greater labeling ($p<.001$) of brain cholesterol compared to {2-^{14}C}glucose; whereas the {2-^{14}C}glucose injected animals showed ca. fourfold greater labeling ($p<.001$) of brain proteins than {3-^{14}C}D(-)-3-hydroxybutyrate at all time points. The difference in labeling of brain glycolipids and phospholipids was less striking, but greater labeling was apparent at each time point in the {2-^{14}C}glucose injected animals compared to the {3-^{14}C}D(-)-3-hydroxybutyrate injected animals. These data suggest that D(-)-3-hydroxybutyrate behaves differently than glucose, being a more direct precursor for brain cholesterol biosynthesis and a less effective precursor for brain protein synthesis. Further studies ascertaining the specific activities of the precursors are necessary to quantitate the respective contributions of D(-)-3-hydroxybutyrate and glucose to lipid and protein synthesis in the rat brain during development.

INTRODUCTION

Evidence is accumulating which supports the contention that D(-)-3-hydroxybutyrate (βOHB) is a potential oxidizable substrate for cerebral metabolism both in man (1,2) and in animals (3). Uptake by the rat brain is related linearly to the arterial concentration of the ketone body (4) and seems to be a major precursor for the synthesis of brain dicarboxylic amino acids during early development (5). The role of βOHB as a precursor for certain structural constituents in the developing rat brain has received less attention. In this study we compared {3-^{14}C}βOHB to {2-^{14}C}glucose as potential precursors for brain proteins and lipids in the 15-day old Sprague-Dawley rat.

EXPERIMENTAL PROCEDURES

Litter sizes initially were reduced to eight pups and then left undisturbed until 15 days of age. The animals were injected subcutaneously (0.25 μc/gm body wt) with either {2-^{14}C}glucose (11.6 μc/μmole) or {3-^{14}C}βOHB (11.3 μc/μmole) and returned to their mothers. The animals were killed by decapitation at 3, 6, and 24 hr. The brains rapidly were removed and frozen on dry ice. The frozen tissue was stored at -80 C until extracted. The brain wt in the two groups did not differ significantly. Lipids were extracted according to the method of Folch et al. (6) and fractionated into cholesterol, glycolipids, and phospholipids by silicic acid column chromatography according to Rouser et al. (7). These lipid fractions were analyzed by the following methods: cholesterol, Zlatkis et al. (8); galactose, Hess and Lewin (9); and lipid phosphorus, Rouser et al. (10). The residue from the lipid extractions was used to prepare total proteins according to Agrawal et al. (11). Protein was measured according to Lowry et al. (12). The radioactivities in the brain total proteins, cholesterol, glycolipids, and phospholipids were determined with a Model 3375 Packard spectrometer. The efficiency was 87% for the protein samples prepared for counting (11). The fractionated lipid samples were dissolved in chloroform-methanol (2:1 v/v) and counted in fluid containing 4 g Omnifluor in 1000 ml toluene at an efficiency of 90%.

The animals injected with {2-^{14}C}glucose demonstrated ca. fourfold heavier labeling of

TABLE I

Incorporation of [^{14}C] Label into Brain Proteins and Lipids after the Intraperitoneal Injection of [2-^{14}C] Glucose or [3-^{14}C] D(-)-3-Hydroxybutyrate (βOHB)[a]

Time (hr)	Precursor (N)[b]	Protein	Cholesterol	Glycolipid	Phospholipid
3	[2-^{14}C] Glucose (3)	1531 ± 11[c]	1807 ± 12[c]	2292 ± 230[d]	1660 ± 44[e]
	[3-^{14}C] βOHB (3)	364 ± 6	3316 ± 100	1290 ± 172	1295 ± 48
6	[2-^{14}C] Glucose (2)	1464 ± 25[c]	1575 ± 77[c]	2555 ± 218[d]	1555 ± 95
	[3-^{14}C] βOHB (3)	335 ± 7	3294 ± 40	1439 ± 141	1365 ± 27
24	[2-^{14}C] Glucose (2)	1109 ± 81[c]	1362 ± 57[c]	2542 ± 7[e]	1658 ± 228
	[3-^{14}C] βOHB (2)	263 ± 24	2988 ± 56	859 ± 28	1161 ± 62

[a]Data expressed as cpm/mg (mean ± SEM).
[b](N) = number of animals.
[c]$p < .001$.
[d]$p < .025$.
[e]$p < .005$.

brain total proteins compared to the {3-^{14}C}βOHB injected group ($p<.001$) (Table I). The distribution of the ^{14}C label among the lipid fractions also differed significantly, depending upon the injected radioactive precursor (Table I). The labeling of brain cholesterol by {3-^{14}C}βOHB far exceeded that of {2-^{14}C}glucose ($p<.001$). The glycolipid and phospholipid fractions were more heavily labeled when {2-^{14}C}glucose was used as the precursor.

DISCUSSION

These data demonstrate that βOHB is an excellent precursor for brain cholesterol in the 15-day old suckling rat. It is well known that rat milk is high in fat and that suckling pups are ketotic for this reason. The available evidence would suggest that βOHB, after being extracted from the blood by brain, is oxidized to acetoacetate by the mitochondrial enzyme, D-β-hydroxybutyrate dehydrogenase (E.C.1.1.1.30.) (13). This enzymatic reaction presumably is the principal pathway for metabolism of βOHB. It seems logical to suggest that the intramitochondrial acetyl CoA pool should be more heavily labeled from a direct precursor, such as βOHB, as opposed to glucose, since many alternate pathways are available for the metabolism of glucose before entering the intramitochondrial acetyl CoA pool. Acetyl CoA condenses with oxaloacetate to form citrate. Citrate presumably is transported across the mitochondrial membrane and cleaved enzymatically to acetyl CoA and oxaloacetate to provide the cytoplasmic precursor for cholesterol and fatty acid synthesis (14). This reasoning may explain the heavier labeling pattern of brain cholesterol in the {3-^{14}C}βOHB injected rats.

Similar reasoning might lead one to anticipate heavier labeling of brain phospholipids by {3-^{14}C}βOHB as well, which was not observed. Systemically administered {3-^{14}C}βOHB does not label blood or brain glucose significantly (5) and, as such, would not be expected to act as a precursor for the galactose or glycerol moieties of brain glycolipids or phospholipids, respectively. Rather, {3-^{14}C}βOHB might be expected to label the fatty acid moieties of these two brain lipid fractions with the respective fatty acid moieties being derived from a common brain acetyl CoA pool. The similarity between the cholesterol-phospholipid ratio (2.56) and the cholesterol-glycolipid ratio (2.57) at the 3 hr time point in the {3-^{14}C}βOHB injected animals helps support this contention. Assuming a similar cholesterol-fatty acid ratio of the ^{14}C label in the brain glycolipids and phospholipids of the {2-^{14}C}glucose injected animals, it would appear that a significant portion of the radioactivity in the glycolipid and phospholipid fractions resides in the galactose and glycerol moieties respectively.

Alternatively, the heavier labeling of brain cholesterol by {3-^{14}C}βOHB could suggest that the cytoplasmic precursors of cholesterol synthesis are not necessarily derived exclusively from the intramitochondrial citrate pool. Acetoacetate is activated intramitochondrially to acetoacetyl CoA by 3-oxoacid CoA transferase (E.C.2.8.3.5.). Our studies on the subcellular distribution of D-β-hydroxybutyrate dehydrogenase and 3-oxoacid CoA transferase of 15-day old rat brain demonstrate that these enzymes are localized exclusively in mitochondria (Agrawal and DeVivo, manuscript in preparation). Our enzymatic study also confirms the observation of others in both brain (14) and

liver (15) that acetoacetyl CoA thiolase (E.C. 2.3.1.9.) is present both in mitochondrial and cytoplasmic fractions. On the basis of these observations, it is reasonable to suggest that βOHB must be oxidized to acetoacetate and acetoacetate activated to acetoacetyl CoA within mitochondria. If acetoacetyl CoA were transported to the cytosol, it could serve as an immediate precursor in the synthesis of 3-hydroxy-3-methylglutaryl CoA (16). This possibility would explain the preferential labeling of the brain cholesterol fraction by {3-^{14}C}βOHB.

An additional unresolved point is the protein labeling by each precursor. Previous studies in the newborn rat have shown heavier labeling of the brain dicarboxylic amino acids 20 min after intraperitoneal injection of {3-^{14}C}βOHB compared to {2-^{14}C}glucose (5). This fact is most apparent in the 15-day old rat brain (DeVivo, Leckie, and Agrawal, manuscript in preparation). Nevertheless, βOHB appears to be a poor precursor for labeling brain protein. It may well be that βOHB is labeling a dicarboxylic amino acid pool which is not directly in continuity with the brain protein synthesizing mechanisms, as opposed to glucose. Further studies are in progress to clarify this point.

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REFERENCES

1. Owen, O.E., A.P. Morgan, H.G. Kemp, J.M. Sullivan, M.G. Herrera and G.F. Cahill, Jr., J. Clin. Invest. 46:1589 (1967).
2. Persson, B., G. Settergen and G. Dahlquist, Acta Paediat. Scand. 61:273 (1972).
3. Hawkins, R.A., D.H. Williamson and H.A. Krebs, Biochem. J. 122:13 (1971).
4. Zivin, J.A., and J.F. Snarr, J. Appl. Physiol. 32:664 (1972).
5. DeVivo, D.C., M.P. Leckie and H.C. Agrawal, Brain Res. 55:485 (1973).
6. Folch, J., M. Lees and G.H. Sloane-Stanley, J. Biol. Chem. 226:497 (1957).
7. Rouser, G., G. Kritchevsky and A. Yamamoto, in "Lipid Chromatographic Analysis," Edited by G. Marinetti, Marcel Dekker Inc., New York, 1967, p. 116.
8. Zlatkis, A., B. Zak and A.J. Boyle, J. Lab. Clin. Med. 41:486 (1953).
9. Hess, H.H., and E. Lewin, J. Neurochem. 12:205 (1965).
10. Rouser, G., A.N. Siakotos and S. Fleischer, Lipids 1:85 (1966).
11. Agrawal, H.C., A.H. Bone and A.N. Davison, Biochem. J. 117:325 (1970).
12. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193:265 (1951).
13. Williamson, D.H., M.W. Bates, M.A. Page and H.A. Krebs, Biochem. J. 121:41 (1971).
14. Middleton, B., Biochem. J. 125:70 (1971).
15. Clinkenbeard, K.D., T. Sugiyama, J. Moss, W.D. Reed and M.D. Lane, J. Biol. Chem. 248:2275 (1973).
16. Sugiyama, T., K. Clinkenbeard, J. Moss and M.D. Lane, Biochem. Biophys. Res. Commun. 48:255 (1972).

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Conversion of Sodium Lithocholate-24-^{14}C to Other Products by Guinea Pig Brain

ABSTRACT

Intracerebral injection of sodium lithocholate into guinea pig showed that this steroid can be converted into other acidic products by the brain, in vivo. One of the products has been identified as 3-keto-5β-cholanoic acid.

It has been shown earlier that 3-keto-5β-cholanoic acid can be converted into other acidic products by guinea pig brain in vitro (1). In vitro conversion of lithocholic acid to other metabolites also has been reported in rats (2). This paper presents evidence for the conversion of lithocholic acid to 3-keto-5β-cholanoic acid, and some other products, by guinea pig brain in vivo.

Sodium lithocholate-24-^{14}C (2.5 mg, 1μc/mg) was purified by preparative TLC and then precipitated as a sodium salt. The product was shown to be pure on thin layer chromato-

liver (15) that acetoacetyl CoA thiolase (E.C. 2.3.1.9.) is present both in mitochondrial and cytoplasmic fractions. On the basis of these observations, it is reasonable to suggest that βOHB must be oxidized to acetoacetate and acetoacetate activated to acetoacetyl CoA within mitochondria. If acetoacetyl CoA were transported to the cytosol, it could serve as an immediate precursor in the synthesis of 3-hydroxy-3-methylglutaryl CoA (16). This possibility would explain the preferential labeling of the brain cholesterol fraction by {3-^{14}C}βOHB.

An additional unresolved point is the protein labeling by each precursor. Previous studies in the newborn rat have shown heavier labeling of the brain dicarboxylic amino acids 20 min after intraperitoneal injection of {3-^{14}C}βOHB compared to {2-^{14}C}glucose (5). This fact is most apparent in the 15-day old rat brain (DeVivo, Leckie, and Agrawal, manuscript in preparation). Nevertheless, βOHB appears to be a poor precursor for labeling brain protein. It may well be that βOHB is labeling a dicarboxylic amino acid pool which is not directly in continuity with the brain protein synthesizing mechanisms, as opposed to glucose. Further studies are in progress to clarify this point.

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REFERENCES

1. Owen, O.E., A.P. Morgan, H.G. Kemp, J.M. Sullivan, M.G. Herrera and G.F. Cahill, Jr., J. Clin. Invest. 46:1589 (1967).
2. Persson, B., G. Settergen and G. Dahlquist, Acta Paediat. Scand. 61:273 (1972).
3. Hawkins, R.A., D.H. Williamson and H.A. Krebs, Biochem. J. 122:13 (1971).
4. Zivin, J.A., and J.F. Snarr, J. Appl. Physiol. 32:664 (1972).
5. DeVivo, D.C., M.P. Leckie and H.C. Agrawal, Brain Res. 55:485 (1973).
6. Folch, J., M. Lees and G.H. Sloane-Stanley, J. Biol. Chem. 226:497 (1957).
7. Rouser, G., G. Kritchevsky and A. Yamamoto, in "Lipid Chromatographic Analysis," Edited by G. Marinetti, Marcel Dekker Inc., New York, 1967, p. 116.
8. Zlatkis, A., B. Zak and A.J. Boyle, J. Lab. Clin. Med. 41:486 (1953).
9. Hess, H.H., and E. Lewin, J. Neurochem. 12:205 (1965).
10. Rouser, G., A.N. Siakotos and S. Fleischer, Lipids 1:85 (1966).
11. Agrawal, H.C., A.H. Bone and A.N. Davison, Biochem. J. 117:325 (1970).
12. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193:265 (1951).
13. Williamson, D.H., M.W. Bates, M.A. Page and H.A. Krebs, Biochem. J. 121:41 (1971).
14. Middleton, B., Biochem. J. 125:70 (1971).
15. Clinkenbeard, K.D., T. Sugiyama, J. Moss, W.D. Reed and M.D. Lane, J. Biol. Chem. 248:2275 (1973).
16. Sugiyama, T., K. Clinkenbeard, J. Moss and M.D. Lane, Biochem. Biophys. Res. Commun. 48:255 (1972).

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Conversion of Sodium Lithocholate-24-^{14}C to Other Products by Guinea Pig Brain

ABSTRACT

Intracerebral injection of sodium lithocholate into guinea pig showed that this steroid can be converted into other acidic products by the brain, in vivo. One of the products has been identified as 3-keto-5β-cholanoic acid.

It has been shown earlier that 3-keto-5β-cholanoic acid can be converted into other acidic products by guinea pig brain in vitro (1). In vitro conversion of lithocholic acid to other metabolites also has been reported in rats (2). This paper presents evidence for the conversion of lithocholic acid to 3-keto-5β-cholanoic acid, and some other products, by guinea pig brain in vivo.

Sodium lithocholate-24-^{14}C (2.5 mg, 1μc/mg) was purified by preparative TLC and then precipitated as a sodium salt. The product was shown to be pure on thin layer chromato-

FRACTION NO	CPM	% of total count	CPM	% of total count	
					SOLVENT FRONT
6	166	2.1 %	116	1.2 %	
5	336	4.2 %	559	5.7 %	3-KETO-5β-CHOLANOIC ACID
4	14	0.17 %	111	1.1 %	
3	6,928	87.7 %	7,863	81.2 %	LITHOCHOLIC ACID
2	71	0.9 %	589	6.0 %	
1	11	0.18%	86	0.88%	
0	370	4.6%	356	3.6 %	ORIGIN
	2 hr after injection		48 hr after injection		STANDARDS

FIG. 1. Intracerebral injection of sodium lithocholate-24-^{14}C into guinea pig. Results of thin layer chromatogram (TLC) radioactivity scan of the acidic material obtained from the brain after 2 hr and 48 hr of the injections. The results presented were obtained on 1 animal at a time. In repeat studies the radioactivity in fraction 5 varied from 3.9 to 4.7% in 2 hr experiments and from 4.9 to 5.9% in 48 hr experiments. At 0 hr, region 5 of a TLC had 11 cpm against 324,000 cpm in the lithocholic acid region.

grams (TLC) and also on TLC and gas liquid chromatogram (GLC) of its methyl ester. Sodium lithocholate-24-^{14}C then was injected intracerebrally as described earlier (3). Two animals were sacrificed after 2 hr and two after 48 hr. The brains were homogenized and the homogenate was hydrolyzed with 1% methanolic NaOH and the acidic products obtained as usual.

A quarter of the acidic product was subjected to TLC (trimethyl pentane, ethyl acetate, acetic aicd, 75:45:1). Figure 1 shows radioactivity scans of TLC on the acidic products at 2 and 48 hrs. In the 2 hr experiment 88% of the radioactivity was found in the region where monohydroxy bile acids, including the starting material, would be located (R_f 0.21 to 0.31). The rest of the radioactivity is located mainly at the origin (di- and tri-hydroxy bile acids) and in the less polar regions of the chromatogram. TLC of 1/2 product of the 48 hr experiment shows a very similar profile of radioactivity. An autoradiograph of the 2 hr experiment was obtained by superimposing an X-ray film over a developed TLC and leaving it in the dark for 8 weeks (Fig. 2). It confirms the

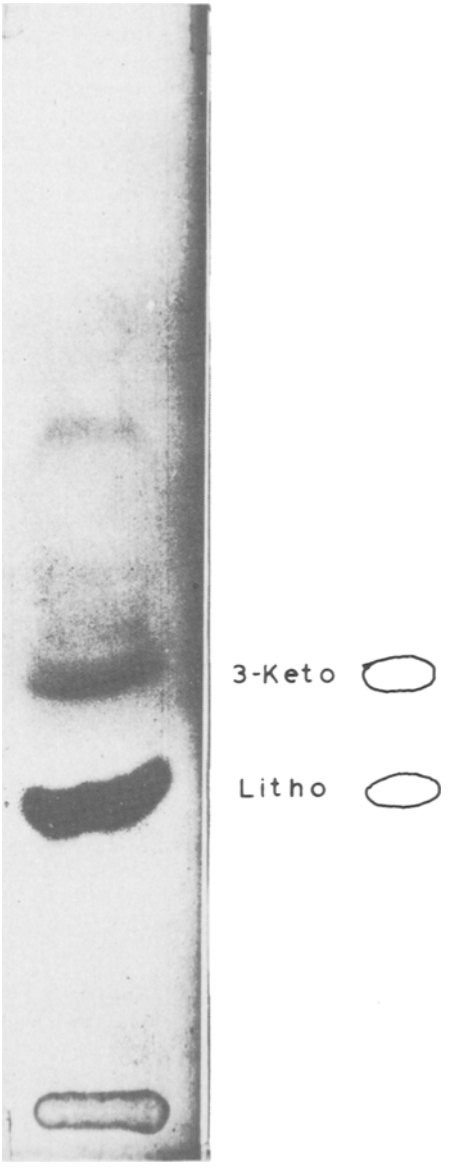

FIG. 2. Intracerebral injection of sodium lithocholate-24-^{14}C into guinea pig. Autoradiograph of a thin layer chromatogram (TLC) of the acidic material obtained from the 2 hr experiment.

results of the radioactivity scan and shows that one of the less polar products had the same mobility on TLC (R_f 0.42) as 3-keto-5β-cholanoic acid.

The remaining product from the 2 hr experiment was subjected to preparative TLC. Areas corresponding to fractions 0, 3, 5, and 6 of the earlier TLC (Fig. 1) were scraped separately and eluted with methanol-ether, 9:1; fraction 5 was cochromatographed with standard 3-keto-5β-cholanoic acid. To the material from fraction 5

was added 10 mg 3-keto-5β-cholanoic acid, and it was methylated with BF_3-methanol. TLC and radioactivity scanning of the methylated material showed that the radioactivity stayed in the methyl 3-keto-5β-cholanoate region. Crystallization of methylated product with methanol did not remove the radioactivity from the crystals, and it remained constant during three subsequent crystallizations (37-39 cpm/mg) confirming the presence of labeled 3-keto-5β-cholanoic acid.

The amount of radioactivity in the isolated fractions was relatively small, which is understandable in view of our earlier observation (3) that the injected bile salt is transported out of the brain within a few hrs. (As can be seen from the results presented above, only 0.050% of the injected dose was recoverable from the brain after 2 hrs). Also the percentages of the injected bile salt and its products are not conclusive, since the rate of their transportation out of the brain in relation to lithocholic acid is not known. This study does, however, suggest that the guinea pig brain has the enzymatic capacity to convert lithocholic acid to 3-keto-5β-cholanoic acid and to some more polar products.

3-Hydroxy steroid dehydrogenase and 6β-hydroxylase activity have been found in adult rat brains (2). Our study shows that a similar activity is present in the guinea pig brain. Even though the more polar product(s) seen in fraction O of the chromatogram has not been identified, it can be speculated that it is an oxygenated derivative of the injected bile salt or of a product and could be a compound similar to the 3α, 6β-dihydroxy-5β-cholanoic acid identified in rats (2).

The radioactivity in fraction 3 of the chromatogram could be due to more than one compound. However, the presence of isolithocholic acid, a compound which could arise by the further metabolism of 3-keto-5β-cholanoic acid (4) can be ruled out, since that acid separates from lithocholic acid on TLC in the solvent systems used here (4).

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REFERENCES

1. Naqvi, S.H.M., B.L. Herndon, M.T. Kelley, V. Bleisch, R.T. Aexel and H.J. Nicholas, J. Lipids Res. 10:115 (1969).
2. Martin, C.W., and H.J. Nicholas, Steroids, 19:549 (1972).
3. Naqvi, S.H.M., B.L. Herndon, L. Del Rosario and H.J. Nicholas, Lipids, 5:964 (1970).
4. Naqvi, S.H.M., and H.J. Nicholas, Steroids, 16:297 (1970).

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Preparation of Fatty Acid Methyl Esters from Triglycerides on Thin Layer Plates

ABSTRACT

A simple and convenient procedure for the preparation of methyl esters from triglycerides with potassium methoxide (KOMe) as catalyst on thin layer chromatoplates has been described. The triglyceride was separated and the reaction was carried out on a thin layer chromatoplate without extraction of triglyceride. A final extraction with CS_2 was sufficient for injection of the product into a gas-chromatograph. The method has been tested with coconut, peanut, and watermelon seed oils. The method is applicable to 10-20 mg of oil sample and is rapid.

INTRODUCTION

With the introduction of gas liquid chromatography (GLC) for the analysis of lipids, the preparation of methyl esters from milligram quantities of lipid samples by quicker methods has gained interest. Among various methods for this conversion (1-8, 10,11), the procedure by Luddy et al. (9) using methanol and catalytic amounts of KOMe is used widely.

This paper describes a procedure for the separation of the triglycerides from the oil in the form of a band on thin layer plate and subsequent conversion to methyl esters on the same plate with methanol and KOMe as catalyst. The method has been tested with coconut,

was added 10 mg 3-keto-5β-cholanoic acid, and it was methylated with BF_3-methanol. TLC and radioactivity scanning of the methylated material showed that the radioactivity stayed in the methyl 3-keto-5β-cholanoate region. Crystallization of methylated product with methanol did not remove the radioactivity from the crystals, and it remained constant during three subsequent crystallizations (37-39 cpm/mg) confirming the presence of labeled 3-keto-5β-cholanoic acid.

The amount of radioactivity in the isolated fractions was relatively small, which is understandable in view of our earlier observation (3) that the injected bile salt is transported out of the brain within a few hrs. (As can be seen from the results presented above, only 0.050% of the injected dose was recoverable from the brain after 2 hrs). Also the percentages of the injected bile salt and its products are not conclusive, since the rate of their transportation out of the brain in relation to lithocholic acid is not known. This study does, however, suggest that the guinea pig brain has the enzymatic capacity to convert lithocholic acid to 3-keto-5β-cholanoic acid and to some more polar products.

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REFERENCES

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This paper describes a procedure for the separation of the triglycerides from the oil in the form of a band on thin layer plate and subsequent conversion to methyl esters on the same plate with methanol and KOMe as catalyst. The method has been tested with coconut,

TABLE I

Major Component Acids (% wt.) of Triglycerides from Oils

Component acid	Watermelon oil		Peanut oil		Coconut oil	
	Method A[a]	Method B[b]	Method A[a]	Method B[b]	Method A[a]	Method B[b]
C_8					6.98	7.04
C_{10}					5.70	5.94
C_{12}					45.87	47.84
C_{14}					20.00	21.00
C_{16}	13.14	15.14	8.98	9.32	8.90	7.91
C_{18}	8.55	7.32	5.50	5.44	2.78	2.64
$C_{18:1}$	18.84	18.82	58.62	57.77	8.37	6.77
$C_{18:2}$	59.47	58.66	25.24	26.10	1.40	0.86
$C_{18:3}$			0.66	0.61		
C_{20}			1.00	0.76		

[a]Method of Luddy et al. (9).
[b]Present method (60 C).

peanut, and watermelon seed oils; and the results have been compared with those obtained by Luddy's (9) procedure. The method has been found to be rapid, simple, and suitable for the routine GLC analysis. The method is applicable to small sample size and eliminates many manipulations.

EXPERIMENTAL PROCEDURES

About 20-25 mg of oil in petroleum ether (40-60 C) was applied as a band on 14 x 14 cm silica gel plate of 0.5 mm. thickness. The plate was developed in an S-chamber using the solvent mixture benzene, ether, and acetic acid (100:10:0.5). The developed plate was lightly sprayed with Rhodamine-6G and the triglyceride band marked under UV light. The plate was heated on a tray in an oven at 60 C for about 5 min. The tray was taken out, and 0.5N KOMe in methanol was added immediately throughout the triglyceride band from a capillary dropper. After ca. a minute the band was scraped off in a small beaker (25 c.c.); 0.5 gm of fused $CaCl_2$ powder was added, and extracted with CS_2 (total volume 3-4 c.c.). The CS_2 was evaporated under nitrogen, and the percentage composition of the methyl esters was evaluated by GLC.

For comparison the triglyceride band separated as above was extracted with $CHCl_3$. The solvent was removed under vacuum, and the triglycerides obtained were subjected to esterification according to Luddy et al. (9) and finally analyzed by GLC.

RESULTS AND DISCUSSION

The results in Table I indicate that the present procedure gives data comparable to Luddy's method (9). The oils of coconut, peanut, and watermelon seed have been chosen to cover a wide variations in fatty acid compositions.

Experiments at room temperature showed good results with watermelon triglycerides whereas some significant differences occurred in the case of the other two oils.

Higher plate temperature at the time of application of the reagent increased the efficiency of the conversion. Studies on the effect of heating the plate at various temperatures up to 80 C with 1 min reaction time indicated that a temperature of 60 C was most suitable for the conversion, whereas the possibility of losing C_8- and lower acids increased rapidly at temperatures above 70 C. Similar losses in the lower fatty acids in the case of coconut oil resulted, even at room temperature when the reaction time was increased up to 1 hr by keeping the treated plates in methanol saturated desiccator. The average time from the application of the sample on the TLC plate to the extraction of methyl esters is ca. 40 min, and the yield of fatty acids is ca. 75% of the oil. Studies on this reaction with different standard triglycerides are in progress and will be published elsewhere.

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REFERENCES

1. Craig, B.M., and N.L. Marty, JAOCS 36:549 (1959).

2. Hyun, S.A., G.V. Vahouny and C.R. Treadwell, Anal. Biochem 10:193 (1965).
3. Kishimoto, Y., and N.S. Radin, J. Lipid Res. 6:435 (1965).
4. McGinnis, G.W., and L.R. Dugan, Jr., JAOCS 42:305 (1965).
5. Roper, R., and T.S. Ma, Michrochem. J. 1:245 (1957).
6. Sato, Y., and M. Momotani, Bunseki Kagaku, 10:196 (1961).
7. Smith, L.M., J. Dairy Sci. 44:607 (1961).
8. Tove, S.B., J. Nutr. 25:361 (1961).
9. Luddy, F.E., R.A. Barford, S.F. Herb and P. Magidman, JAOCS 45:549 (1968).
10. Luddy, F.E., R.A. Barford and R.W. Riemenschneider, JAOCS 37:447 (1960).
11. Morrison, W.R., and L.M. Smith, J. Lipid Res. 5:600 (1964).

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Fatty Acid Composition of Lipids from *Saccharomyces fragilis*

ABSTRACT

Lipids extracted from *Saccharomyces fragilis*, grown in whey and deproteinized whey, were similar in amount and fatty acid composition. On a dry weight basis, the yeasts contained 3.0% total lipid and 0.5% saponifiable lipid. The fatty acids identified by gas chromatography-mass spectrometry were 2.5% C14:0, 19.2% C16:0, 11.9% C16:1, 1.28% C16:2, 3.4% C18:0, 27.0% C18:1, 25.1% C18:2, and 9.6% C18:3 with less than 1% of the following fatty acids present: C10:0, C12:0, C14:1, C15:0, and C17:0.

INTRODUCTION

Saccharomyces fragilis, a lactose fermenting yeast, has been shown efficient in producing single cell protein from whey (1,2). Vrignaud (3) reported the amino and fatty acid composition of *S. fragilis Jorgenson* and *S. lactis Dombrowski,* which were grown on deproteinized whey. In this paper we report the determination of total and saponifiable lipids and the fatty acid composition of *S. fragilis,* grown in whey and in whey from which the serum proteins had been removed.

EXPERIMENTAL PROCEDURES

S. fragilis culture used in this research was isolated originally from a lactase preparation (Control No. 5850), supplied by the Nutritional Biochemical Corp., Cleveland, Ohio. Stock cultures were maintained on yeast-lactose agar slants and transferred to lactose broth for a 16 hr incubation in a shaker bath at 32 C before inoculation in the whey medium.

For single cell protein production, the cultures were grown in a cyclone-type fermenter on standard whey or on deproteinized whey. For the preparation of whey from skim milk, casein was precipitated by acidification with lactic acid to a pH of 4.4-4.6 and removed by filtration through Whatman No. 1 paper. The whey was sterilized for 15 min at 121 C. For the deproteinized whey substrate, the coagulated proteins were removed from the sterilized whey by aseptically filtering through Whatman No. 1 paper.

An inoculum of 2.4 g yeast (on a dry wt basis) was introduced to 1600 ml whey, supplemented with 5% corn steep liquor, 0.5% $(NH_4)_2HPO_4$, and 0.1% $FeNH_4(SO_4)_2$. The culture was aerated at 32 C for eight to nine hr at a flow rate of 600 ml/min with air sterilized by filtration through a Millipore membrane, pore size 0.22μ. The yeast was harvested by centrifugation, washed twice with distilled water and freeze-dried.

For determination of total lipid content, 2 g aliquots of lyophilized yeast were stirred for 16 hr in 80 ml chloroform-methanol (2:1) under nitrogen. The nonlipid material was removed by rinsing the lipid extract, as described by Folch et al. (4). The solvent was removed by aspiration and the total lipid determined by weighing the dried extract. The lipid extract was then saponified in 40 ml methanol and 150 mg KOH for 5 hr under nitrogen. After removal of the nonsaponifiable lipid by petroleum ether, the saponified extract was acidified and the free fatty acids (FFA) extracted with petroleum ether. The sample was dried and weighed to determine the amount of saponifiable lipids.

The FFA (0.10 g-0.15 g), dissolved in 0.5 ml benzene, were methylated by refluxing in 5 ml anhydrous 0.5 N $NaOCH_3$ for 15 min. Five milliliters of distilled water were added to the methylation mixture and the methyl esters extracted in two 10 ml rinses of petroleum

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TABLE I

Fatty Acid Composition of Yeasts Grown on Whey

Fatty acid identification	Relative fatty acid composition of *Saccharomyces fragilis*[a] (area %)	Relative fatty acid composition of *S. fragilis Jorgenson* and *S. lactis Dombrowski*[b] (wt%)
4:0	—	8.49
10:0	Trace	—
12:0	<1	0.94
14:0	2.52	1.98
14:1	<1	—
15:0	<1	0.94
16:0	19.19	19.63
16:1	11.92	6.37
16:2	1.28	—
17:0	Trace	0.94
17:1	—	2.12
18:0	3.40	3.02
18:1	26.97	24.02
18:2	25.13	18.22
18:3	9.64	6.66

[a]Average of five runs of two batches of yeast prepared in whey and three runs of one batch of yeast prepared in whey with serum proteins removed.

[b]From results of Vrignaud (3) for cultures grown on deproteinized whey.

ether and dried over Na_2SO_4 for 5 min.

One microliter injections of the concentrated extracts were examined on a Varian Aerograph 1400 gas chromatograph (GC) equipped with a flame ionization detector and a 9 ft x 1/8 in. O.D. stainless steel column packed with 15% diethyleneglycolsuccinate (DEGS) on Chromosorb W (80/100 mesh). The GC was operated isothermally at 180 C with a carrier gas flow rate of 20 ml/min. Peaks were quantified by triangulation and identified by comparison of retention times with those of authentic standards or by interpretation of their mass spectra. For mass spectrometric examination of the esters, a Pye 150 GC equipped with a 5 ft x 1/4 in. O.D. glass column, packed with 15% DEGS on Chromosorb W, was interfaced with a membrane separator operating at 180 C into beam 2 of an AEI-MS-30, double beam, double focusing mass spectrometer. Perfluorokerosene served as a marker in beam 1. The ion source voltage was maintained at 70 eV. GC was programed at 4 C/min from 110-160 C.

RESULTS

On a dry wt basis, the total lipid and saponifiable lipid contents of *S. fragilis* grown in both wheys were 3.0 and 0.5%, respectively. In Table I the fatty acid composition of *S. fragilis* is compared with those of the lactic yeasts reported by Vrignaud (3). There was no difference at the 5% level of significance in the fatty acid composition of different batches of yeast grown under the same conditions or of yeasts grown in whey or deproteinized whey. In this investigation butyric acid was not present in detectable amounts as shown by GC analysis of yeast lipids esterified by the method of Shehata et al. (5) which permits the recovery of short chain fatty acids.

The presence of hexadecadienoic acid in *S. fragilis* seems unique among yeasts. It has not been found in two other yeasts, *S. cerevisiae* (6) and *Rhodotorula gracilis* (7), in the yeast-like fungus, *Pullularia pullulans* (8), or in either *S. fragilis Jorgenson* or *S. lactis Dombrowski* (3). The overall composition of *S. fragilis* is quite high in di- and triunsaturated C_{18} fatty acids, similar to the fatty yeast, *Rhodotorula gracilis.*

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REFERENCES

1. Wasserman, A.E., W. Hopkins and N. Porges, Sewage Ind. Wastes 30:913 (1958).

2. Wasserman, A.E., J. Dairy Sci. 43:1231 (1960).
3. Vrignaud, Y., Rev. Inst. Pasteur Lyon 4:147 (1971).
4. Folch, J., M. Lees and G.H. Stanley, J. Biol. Chem. 226:497 (1957).
5. Shehata, A.Y., J.M. deMan and J.C. Alexander, Can. Inst. Food Technol. J. 3:85 (1970).
6. Suomalainen, H., and T. Nurminen, Chem. Phys. Lipids 4:247 (1970).
7. Kessel, R.H.J., J. Appl. Bact. 31:220 (1968).
8. Merdinger, E., and C. Cwiakala, Lipids 2:276 (1967).

[Received July 23, 1973]

Lipid Composition of the Pecan Weevil, *Curculio caryae* (Horn)

ABSTRACT

The lipids of larvae, male adults, and female adults of the pecan weevil, *Curculio caryae* (Horn), were studied, and special attention was given the fatty acid composition. The larvae contained an unusually high amount of lipid material (40.6%), most of it concentrated in the neutral lipid fraction. Male and female adults contained more conventional quantities, 5.8 and 8.2%, respectively. Oleic acid was the major fatty acid in the total and neutral lipids of all stages; linoleic acid was the most abundant in the phospholipid fractions.

INTRODUCTION

The adult pecan weevil, *Curculio caryae* (Horn), attacks green maturing nuts in late summer and damages them by making feeding and oviposition punctures. The insect then undergoes an extended life cycle which takes 2-3 years to complete. The extended life cycle of the pecan weevil indicates that the role of lipids in this insect is a central one. The importance of lipids in the biochemistry of insects has been stressed in recent reviews (1,2). Also, extensive studies in the order Coleoptera have been conducted (3-6). Our investigation was designed to add to the body of general knowledge of insect lipids and specifically to further the understanding of the lipids of Coleoptera.

The adult pecan weevils were newly-emerged and unfed. The larvae were last instar and about to enter diapause. Extraction, isolation, and identification were as previously reported (5).

DISCUSSION

Table I shows the percentage distribution of the lipids in the stages studied. The total lipid percentage reported for the larvae is among the highest ever reported for any stage of any insect. It results, of course, from the larvae feeding on lipid-rich meats of the pecan, its habitat and sole source of food since hatching. However, the buildup of this lipid reserve also prepares the larva for the long diapause underground during which no feeding occurs. It is noteworthy that 97.6% of the lipid is neutral lipid, most likely in the form of triglyceride, a readily available source of energy. The token amount of phospholipid (2.4%) probably is contained in the membranous structures of the larvae.

The total lipid percentages of the adult male (5.8%) and female (8.2%) fall within the normal range for most newly-emerged, unfed insects with their lipid stores depleted by the long dormant period in the soil. Females contain a slightly larger amount of lipid, which is a common occurrence in other Coleoptera and also other orders of insects. Presumably, this extra load of fat is used for reproductive purposes. In both males and females, over 90% of the total lipid is in the form of neutral lipids.

The fatty acid composition of the lipids of the pecan weevil is shown in Table II. The fatty acid profile of the total lipids is remarkably consistent for the three states of the insect. Oleic acid is the most abundant fatty acid, and the percentage of oleic acid in these weevils exceeds that of most other insects (2), probably a reflection of dietary influences (7). The predominance of oleic acid also is carried over into the neutral lipid fraction, which is not surprising since over 90% of the total lipid is neutral lipid. The predominant fatty acid in the phospholipid fraction is linoleic acid (50%). Similar large quantities of linoleic acid and other highly unsaturated acids in the phospho-

TABLE I

Weight Relationships of Pecan Weevil Lipids

Developmental stage	Total lipid (%)	Neutral lipid (% of total)	Phospholipid (% of total)
Larvae	40.6	97.6	2.4
Adult male	5.8	91.2	8.8
Adult female	8.2	95.0	5.0

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3. Vrignaud, Y., Rev. Inst. Pasteur Lyon 4:147 (1971).
4. Folch, J., M. Lees and G.H. Stanley, J. Biol. Chem. 226:497 (1957).
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6. Suomalainen, H., and T. Nurminen, Chem. Phys. Lipids 4:247 (1970).
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Adult female	8.2	95.0	5.0

TABLE II

Fatty Acid Composition of Total Lipid, Neutral Lipid, and Phospholipid of the Pecan Weevil

Developmental stage and lipid class	Fatty acids as mole % of total fatty acids					
	16:0	16:1	18:0	18:1	18:2	18:3
Larvae						
Total lipid	2.0	Trace	Trace	68.1	29.7	Trace
Neutral lipid	2.9	Trace	1.4	61.3	34.2	Trace
Phospholipid	8.0	Trace	4.0	35.5	49.0	3.2
Male adults						
Total lipid	3.7	Trace	Trace	66.9	29.2	Trace
Neutral lipid	3.6	Trace	Trace	68.6	27.6	Trace
Phospholipid	4.7	0.8	1.9	24.2	50.0	18.1
Female adults						
Total lipid	3.3	Trace	Trace	66.1	30.5	Trace
Neutral lipid	4.0	Trace	Trace	60.8	35.1	Trace
Phospholipid	3.4	Trace	3.2	17.2	49.3	26.7

lipids has been observed in *Anthonomus grandis* (6) and a variety of other insects (2). Linolenic acid was present in measurable amounts only in the phospholipid fractions; the concentration was highest in female adults. Insects are known to be unable to synthesize polyunsaturated fatty acids (8), and linoleic and linolenic acids are essential to most insects for normal growth or reproduction. The predominance of these acids in the phospholipids of the pecan weevil and other insects, therefore, is important and worthy of further study.

The phospholipids tentatively identified in the pecan weevil were phosphatidyl ethanolamine, phosphatidyl choline, sphingomyelin, lysophosphatidyl choline, and cardiolipin. Phosphatidyl ethanolamine was the most abundant phospholipid present in the larvae, and phosphatidyl choline was the most abundant in the adults.

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REFERENCES

1. Gilbert, L.I., Advan. Insect Physiol. 4:69 (1967).
2. Fast, P.G., Prog. Chem. Fats II. 18:181 (1970).
3. Fast, P.G., Lipids 1:209 (1966).
4. Rao, K.D.P., and H.C. Agrawal, Comp. Biochem. Physiol. 30:161 (1969).
5. Henson, R.D., A.C. Thompson, R.C. Gueldner and P.A. Hedin, Lipids 6:352 (1971).
6. Henson, R.D., A.C. Thompson, R.C. Gueldner and P.A. Hedin, J. Insect Physiol. 18:161 (1972).
7. French, R.B., JAOCS 39:176 (1962).
8. Lambremont, E.N., C.I. Stein and A.F. Bennet, Comp. Biochem. Physiol. 16:289 (1969).

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On the Site of the Microbiological Reduction of Cholesterol to Coprostanol in the Rat[1]

ABSTRACT

The microbiological reduction of the 5-ene bond of cholesterol (cholest-5-en-3β-ol) to form coprostanol (5β-cholestan-3β-ol) occurs in the gastrointestinal tract of many species. Data are presented which show that this activity occurs predominantly in the ceca in cholesterol fed rats. This explains the report by others that the removal of the ceca causes coprostanol to disappear from the feces.

INTRODUCTION

The microbiological modification of intestinal sterols by indigenous microflora has been

[1]Mississippi Agricultural and Forestry Experiment Station Publication No. 2643.

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Fatty Acid Composition of Total Lipid, Neutral Lipid, and Phospholipid of the Pecan Weevil

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Larvae						
Total lipid	2.0	Trace	Trace	68.1	29.7	Trace
Neutral lipid	2.9	Trace	1.4	61.3	34.2	Trace
Phospholipid	8.0	Trace	4.0	35.5	49.0	3.2
Male adults						
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INTRODUCTION

The microbiological modification of intestinal sterols by indigenous microflora has been

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shown to exert a protective effect against tissue cholesterol accumulations in rats fed exogenous cholesterol (1). The microbiological reduction of cholesterol to coprostanol has been shown to be abolished by poorly digested dietary carbohydrates (2) and orally administered antibiotics (3). Eyssen et al. (4) recently has reported that the surgical removal of the cecum of conventional rats results in the disappearance of fecal coprostanol within two days after the cecectomy and presents evidence that the cecum either supplies needed nutrients or bacterial inocula to support the microbial reduction in the large intestine or is the major site of this reduction itself. During the course of investigations on steroid balance in rats, we investigated the amounts of cholesterol and coprostanol in the cecal contents and feces of individual rats.

METHODS

Mature male rats, 120 days old, were fed semipurified diets based upon casein, starch, supplemental vitamins and minerals, 5% corn oil, and 0.5% cholesterol. The rats were housed in wire-bottom cages for the collection of feces. Diet and water were supplied ad libitum. After a 21-day period for adjustment to diet and quarters, fecal samples were collected, the animals sacrificed, and the ceca removed and stored in 95% ethanol for analysis.

Feces and cecal contents were analyzed for cholesterol and coprostanol by the techniques of Miettinen et al. (5), as outlined in our previous paper (6). Differences between means were analyzed by *t* test for groups with different numbers of individuals, according to Snedecor (7).

RESULTS AND DISCUSSION

Table I shows that, as a group and for all individuals for which data are complete, the percentage of coprostanol (mg coprostanol ÷ [mg coprostanol + mg cholesterol] x 100) was greater in the cecal contents than in the feces. Since the cecum is located anterior to the large intestine and empties into it and since no coprostanol is formed in the small intestine and since Eyssen et al. (4) have shown that only part of the diet is retained in the cecum while the rest bypasses it directly to the large intestine, it therefore follows that the coprostanol formed in the cecum and released to the large intestine was diluted by cholesterol bypassing the cecum, resulting in a lower percentage of coprostanol in the feces. It is not possible that this difference in coprostanol content is due to preferential absorption of coprostanol, since this sterol is virtually unabsorbable (8). Swell et al. (9) also have shown that no cholesterol absorption takes place in the large intestine. It is probable that some coprostanol formation did occur in the large intestine in the rat while the microorganisms were in transit; however, Eyssen et al. (4) have shown that after cecectomy this source of fecal coprostanol rapidly disappears. These data prove that, in rats consuming 0.5% cholesterol, the cecum itself was the major site of coprostanol formation and the percentage conversion of cholesterol to coprostanol was more complete in the cecum than in the large intestine.

TABLE I

Percentage of Coprostanol in Cecal Contents and Feces of Male Rats Fed Cholesterol-Containing Diets

Rat	Cecal contents	Feces
1	42.4	4.73
2	26.7	15.3
3	37.1	28.0
4	47.7	4.26
5	35.6	4.52
6	—[a]	2.41
7	—[a]	7.22
8	34.6	4.30
Mean ± S.D.	37.35[b] ± 7.17	8.84[b] ± 8.70

[a]Samples lost.
[b](Sig P < 0.001).

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REFERENCES

1. Kellogg, T.F., Fed. Proc. Fed. Amer. Soc. Exp. Biol. 30:1808 (1971).
2. Kellogg, T.F., and B.S. Wostmann, Biochim. Biophys. Acta 125:617 (1966).
3. Coleman, D.L., and C.A. Baumann, Arch. Biochem. Biophys. 66:226 (1957).
4. Eyssen, H., M. Piessens-Denef and G. Parmentier, J. Nutr. 102:1501 (1972).
5. Miettinen, T.A., E.H. Ahrens, Jr., and S.M. Grundy, J. Lipid Res. 6:411 (1965).
6. Kellogg, T.F., and B.S. Wostmann, Ibid. 10:495 (1969).
7. Snedecor, G.W., "Statistical Methods," Iowa State College Press, Ames, Iowa, 1956, p. 91.
8. Iritani, N., and W.W. Wells, J. Lipid Res. 7:372 (1966).
9. Swell, L., E.C. Trout, Jr., J.R. Hopper, H. Field, Jr., and C.R. Treadwell, J. Biol. Chem. 233:49 (1958).

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Molecular Species of Wax Esters in Jaw Fat of Atlantic Bottlenose Dolphin, *Tursiops truncatus*

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ABSTRACT

The jaw fat of the Atlantic bottlenose dolphin (*Tursiops truncatus*) contains unusual wax esters which can be separated into short chain ($<C_{24}$) and long chain ($>C_{24}$) fractions by thin layer chromatography. The short chain wax esters (28 wt. %) have been characterized as a 72:24:4 mixture of isovaleroyl, isobutyroyl, and 2-methylbutyroyl esters of C_{14}-C_{18} n- and iso-alcohols. The intact $<C_{24}$ esters have been resolved into individual molecular species by gas liquid chromatography on open-tubular polyester columns. The long chain wax esters (12 wt. %) contain C_{10}-C_{22} n- and iso-acids esterified to the same C_{14}-C_{18} n- and iso-alcohols. Gas liquid chromatography of the intact, hydrogenated $>C_{24}$ esters on a short JXR column has characterized them according to carbon number and the number of methyl branches they contain.

INTRODUCTION

The occurrence of isovalerate wax esters of fatty alcohols in the jaw fat of the Atlantic bottlenose dolphin (*Tursiops truncatus*) first was reported in 1930 by Gill and Tucker (1). Isovalerate wax esters have since been reported widely in the head fats of other Delphinidae genera (2,3). Those of *Globicephala melaena* (4-6), *T. gilli* (7), and *Sotalia fluviatilis* (8) have been studied in detail. In their investigation of *T. gilli*, Varanasi and Malins (7) identified a small amount of isoamyl alcohol and concluded that isoamyl wax esters also were present.

As part of our continuing studies on the head fats of the Odontoceti (2, 3, 5, 8-11) to determine if their unusual chemical composition might be related to their role in echolocation (12), we examined the intact wax esters from *T. truncatus* jaw fat using gas liquid chromatography (GLC). The chromatograms obtained showed numerous peaks which eluted in the C_{18}-C_{23} region, some of which did not correspond to either isovaleroyl or isoamyl wax ester standards (10). Further investigation has revealed that both isobutyrate and 2-methylbutyrate wax esters are present in *T. truncatus* jaw fat. This paper presents these findings and describes new GLC techniques for the detailed characterization of molecular species of wax esters in dolphin head fats.

The usual shorthand nomenclature for fatty acids and fatty alcohols is *chain length*: *number of double bonds* ω *position of last double bond relative to terminal methyl group*. In this paper this terminology has been extended to wax esters using the convention *alcohol*|*acid* order.

EXPERIMENTAL PROCEDURES

Materials

The total fatty tissue from inside one mandibular canal of a 1.61 m female *T. truncatus* was supplied by D.K. Caldwell, University of Florida. The sample was dissected from an animal found dead on the beach at Summer Haven, Florida, April 1970. After storage at -20 C for three months, lipid was extracted with chloroform using a Waring Blendor. Thin layer chromatography (TLC) of the extract revealed only a trace of free fatty acid, indicating the absence of any significant lipid hydrolysis in the sample. A small portion of the total fatty oil was hydrogenated fully using PtO catalyst in freshly distilled dioxane (13).

Wax ester standards necessary for the identification of GLC peaks were either synthesized or purchased. The methyl esters of n-11:0, n-12:0, n-13:0, iso-14:0, n-14:0, anteiso-15:0, n-15:0, iso-16:0, n-16:0, 16:1ω7, anteiso-17:0, n-18:0, 18:1ω9, 18:2ω6, 18:3ω3, and 20:1ω9 acids were converted to the analogous isoamyl (iso-5:0) esters by KOH catalyzed alcoholysis or (after $LiAlH_4$ reduction) to the corresponding isovalerate (iso-5:0), isobutyrate (iso-4:0), or 2-methylbutyrate (anteiso-5:0) esters as previously described (10). A mixture of iso-14:0|iso-14:0, iso-14:0|n-14:0, n-14:0|iso-14:0, and n-14:0|n-14:0 wax esters was synthesized by reducing a 1:1 mixture of myristic and 12-methyltridecanoic acids (Analabs, North Haven, Conn.) with $LiAlH_4$, esterifying the resultant alcohols with the original mixture of C_{14} acids using $HClO_4$ as a catalyst (14), then purifying the product by TLC. Other long chain wax ester standards (n-10:0|n-14:0,

TABLE I

Composition of C_4 and C_5 Acids in Short Chain Wax Esters from *Tursiops truncatus* Jaw Fat

Structure	Determined by open-tubular-column GLC[a] of intact wax esters		Determined by packed-column GLC of derived butyl esters	
	Wt. %	Mole %	Wt. %	Mole %
iso-4:0	24.0	26.8	25.5	28.6
iso-5:0	72.2	69.6	74.5	71.4
anteiso-5:0	3.8	3.6		

[a]GLC = gas liquid chromatography.

n-14:0| n-14:0, n-16:0| n-16:0, and n-18:0| n-18:0) were purchased from Analabs.

Methods

The separation of lipid classes by TLC usually was carried out on 200 x 200 mm glass plates coated with a 0.25 mm layer of Adsorbosil-1 (Applied Science Laboratories, State College, Pa.) impregnated with 0.04% rhodamine 6G and developed in petroleum ether:diethyl ether 95:5. Precoated TLC plates (Applied Science Laboratories), developed in hexane:diethyl ether:acetic acid 54:15:1 and then sprayed with rhodamine 6G, gave equivalent results. Plates were examined under UV light, the appropriate lipid bands were scraped off, and the lipids were recovered with diethyl ether. The amount of each lipid class present was measured by IR spectroscopy (2,15).

The wax ester fractions from TLC of the hydrogenated jaw fat were examined by GLC on a 0.53 m packed JXR silicone column, as described in detail elsewhere (9). The short chain wax esters from TLC of the unhydrogenated jaw fat were examined on stainless steel, wall-coated, open-tubular GLC columns, 46 m x 0.25 mm, inside diameter, coated with DEGS (diethyleneglycol succinate), BDS (butanediol succinate), or AP-L (Apiezon-L), essentially as described elsewhere, (9) except that a Perkin-Elmer Model 900 with flame ionization detector (FID) was used. DEGS and BDS columns were operated at 150 C and AP-L at 190 C with appropriate carrier gas inlet pressures. Methyl esters of fatty acids and acetate esters of fatty alcohols were analyzed on the BDS-coated open-tubular column. C_4 and C_5 acids were determined independently by GLC of the butyl esters at 90 C on a 1.83 m x 3 mm inside diameter column packed with 5% SE-30 on 100/120 mesh Gas-Chrom Q (9).

RESULTS AND DISCUSSION

Separation of the hydrogenated *T. truncatus* jaw fat by TLC showed three triglyceride (60 wt. %) and two wax ester (40%) bands, which were identified by comparison with authentic standards in both GLC and TLC analyses. This subfractionation of lipid classes occurs because of the presence of varying ratios of short (C_4 and C_5) and long (C_{10}-C_{22}) chains in the molecules. Triglycerides containing zero, one, and two short chain acids were resolved, and their analysis will be described in a subsequent report. The wax esters, under discussion here, were separated into an upper band where both fatty acid and alcohol were $\geqq C_{10}$ and a lower band where a $\geqq C_{14}$ alcohol was esterified to a C_4 or C_5 acid. These two groups of wax esters subsequently will be referred to as the long chain and short chain wax esters respectively. We previously have demonstrated the TLC separation of such long and short chain wax esters using synthetic mixtures (10). The unhydrogenated jaw fat similarly was resolved into lipid classes.

Recovery and quantitation of the two hydrogenated wax ester bands indicated 28 wt. % short chain and 12% long chain wax esters in the total jaw fat sample. The two wax ester bands from the unhydrogenated fat also were isolated by preparative TLC.

Short Chain Wax Esters

The acids from the short chain wax esters were converted into butyl esters and the alcohols into alcohol acetates. GLC of the butyl esters on a packed 5% SE-30 column revealed two large peaks corresponding respectively to isobutyric (iso-4:0) acid and to isovaleric (iso-5:0) plus 2-methylbutyric (anteiso-5:0) acids (Table I). GLC of the alcohol acetates from the short chain wax esters using open-tubular polyester columns gave the composition reported in Table II. This mixture contained 87.2% saturated C_{14}-C_{18} alcohols, including 34.2% branched (mostly iso), and no polyunsaturated alcohols. Iso-16:0 and n-16:0 were the major components, but appreciable amounts of n-18:0 and 18:1ω9 were present.

TABLE II

Composition of C_9 to C_{22} Fatty Acids and Fatty Alcohols in *Tursiops truncatus* Jaw Fat Wax Esters

Structure	Fatty acids of long chain wax esters[a]	Fatty alcohols (mole %)				
		Long chain wax esters[b]	Short chain wax esters[b]	Iso-5:0 wax esters[c]	Iso-4:0 wax esters[c]	Anteiso-5:0 wax esters[c]
iso-14:0	1.4	—[d]	—	—	—	—
anteiso-14:0	—	trace	—	trace	trace	—
n-14:0	10.1	2.1	1.1	1.9	2.3	4.2
14:1ω9	2.5	—	—	—	—	—
iso-15:0	5.1	8.0	4.8	9.3	7.1	9.8
anteiso-15:0	0.8	trace	0.1	0.2	0.1	—
n-15:0	1.3	3.0	2.7	2.9	3.1	5.0
iso-16:0	8.6	36.1	25.4	34.3	35.1	40.6
anteiso-16:0	—	0.2	0.1	0.2	0.1	—
n-16:0	25.2	37.5	39.2	34.0	35.9	40.4
16:1ω9	4.4	0.2	0.2	0.4	0.3	—
16:1ω7	5.9	[e]	0.3	0.6	0.6	—
16:1ω5	0.2	—	—	—	—	—
iso-17:0	2.5	2.5	3.0	2.6	2.0	—
anteiso-17:0	1.2	1.2	0.6	0.6	1.1	—
n-17:0	4.5	1.1	1.4	1.0	1.1	—
17:1ω8	0.4	—	—	—	—	—
iso-18:0	0.1	0.2	0.2	0.5	0.3	—
anteiso-18:0	—	—	—	0.3	0.1	—
n-18:0	2.0	3.1	8.5	2.9	3.0	—
18:1ω9	5.5	3.2	8.7	5.9	5.4	—
18:1ω7	1.1	1.7	3.6	2.4	2.4	—
18:1ω5	0.1	—	trace	—	—	—

[a]The following additional minor components with percentages also were present in the fatty acids of the long chain wax esters: n-9:0, trace; iso-10:0, 0.5; n-10:0, 2.3; iso-11:0, 1.6; anteiso-11:0, 0.1; n-11:0, 0.2; iso-12:0, 1.0; n-12:0, 3.4; iso-13:0, 1.2; anteiso-13:0, 0.1; n-13:0, 0.4; 18:2ω6, 0.3; 18:3ω3, 0.3; n-20:0, 0.2; 20:1ω11, 0.4; 20:1ω9, 2.1; 20;1ω7, 1.7; 22;1ω13+11, 1.2; 22:1ω9, 0.2.

[b]Determined as alcohol acetates. Long chain implies that both alcohol and acid were $\geq C_{10}$. Short chain, this instance, refers to an alcohol $\geq C_{14}$ esterified to a C_4 or C_5 acid.

[c]Determined as intact wax esters.

[d]Dash indicates no significant amount in appropriate position with sample size adequate to detect 0.1%.

[e]Not visible for technical reasons.

Following our previous experience with the resolution of isomeric short chain wax esters on open-tubular GLC columns (10), we chromatographed the intact short chain wax esters from unhydrogenated *T. truncatus* jaw fat on a 46 m DEGS column (Fig. 1). Comparison of this chromatogram with the acid and alcohol compositions determined above, examination of fractional chain length values (10, 16, 17), and comparison of peak proportions indicated two overlapping patterns. The same distribution pattern found in the isobutyroyl wax ester peaks was repeated in the isovaleroyl wax ester peaks, which eluted slightly later. This is visualized easily by comparing, for example, the iso-16:0|iso-4:0 and n-16:0|iso-4:0 peaks with the pair iso-16:0|iso-5:0 and n-16:0|iso-5:0. The two groups of n-18:0, 18:1ω9, and 18:1ω7 alcohols occurring as both isobutyroyl and isovaleroyl esters are also clearly recognizable.

While most of the peaks in Figure 1 could be identified as esters of the alcohols known to be present with iso-4:0 or iso-5:0 acids, several minor peaks (shaded in Fig. 1) remained which did not fall into either category. Since 2-methylbutyric acid is known to be present in some dolphin head and body fats (6, 18-21), appropriate 2-methylbutyroyl esters of long chain fatty alcohols were synthesized and cochromatographed with the *T. truncatus* short chain wax esters. The unknown peaks and the 2-methylbutyroyl wax ester standards had identical retentions times on DEGS, BDS, and AP-L open-tubular columns, clearly indicating the presence of 2-methylbutyroyl wax esters in the sample. Although the elution patterns of the DEGS and BDS columns were almost identical, the evidence for the 2-methylbutyroyl esters was particularly clearcut on the AP-L column (Fig. 2) where the iso-15:0|iso-5:0 and iso-16:0|iso-4:0 peaks were coincident, but iso-15:0|anteiso-5:0 eluted slightly earlier.

The relative amounts of isobutyric, isova-

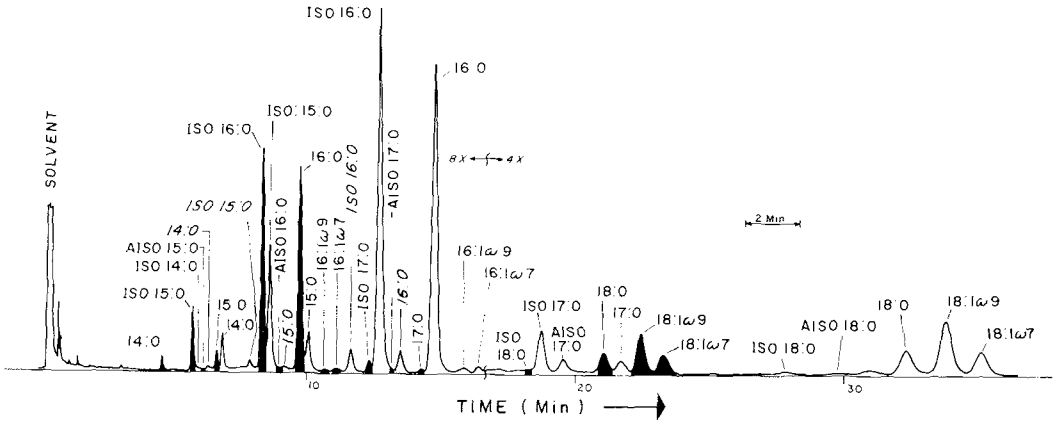

FIG. 1. Gas chromatogram of intact, unhydrogenated, short chain wax esters from *Tursiops truncatus* jaw fat on a wall-coated, stainless steel, open-tubular gas liquid chromatography column, 46 m x 0.25 mm inside diameter, coated with diethyleneglycol succinate polyester. Operating conditions: 150 C, 60 psig He carrier gas, Perkin-Elmer Model 900 gas chromatograph, attenuations 8x and 4x as shown. ■ Isobutyroyl esters, □ Isovaleroyl esters, ▨ 2-Methylbutyroyl esters (*Italics*).

leric, and 2-methylbutyric acids in the short chain wax esters can be calculated directly from the chromatogram in Figure 1. Results (Table I) check closely with those obtained by GLC of the butyl esters, when one realizes that the butyl esters of isovaleric and 2-methylbutyric acids are not resolved by GLC on the packed SE-30 column under the conditions employed in our study.

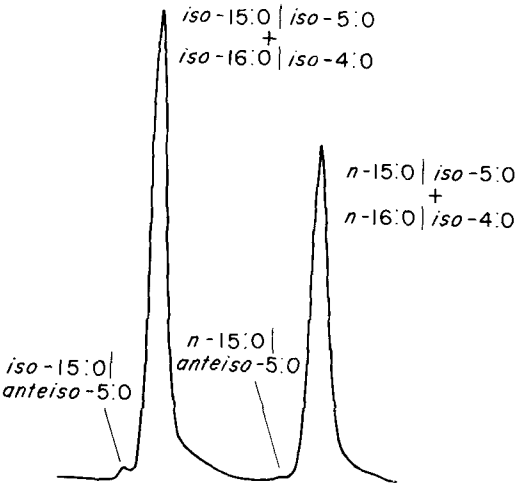

FIG. 2. Portion of gas chromatogram of intact, hydrogenated, short chain wax esters from *Tursiops truncatus* jaw fat on a wall-coated, open-tubular gas liquid chromatography column, 46 m x 0.25 mm inside diameter coated with Apiezon L grease, as modified by the Perkin-Elmer Corporation. These results confirm the presence of 2-methylbutyroyl (anteiso-5:0) wax esters, which elute before the corresponding combined isovaleroyl (iso-5:0) and isobutyroyl (iso-4:0) isomers. Operating conditions: 190 C, 80 psig He carrier gas, Perkin-Elmer Model 900 gas chromatograph, attenuation 2x.

The compositions of the fatty alcohols esterified to isobutyric, isovaleric, and 2-methylbutyric acids were calculated from the open-tubular GLC analysis of both unhydrogenated and hydrogenated short chain wax esters. Results (Table II) show almost identical alcohol mixtures in the isobutyroyl, isovaleroyl, and 2-methylbutyroyl esters, indicating that the alcohols were probably all drawn from a common metabolic pool. Since only the major 2-methylbutyroyl esters could be determined from our chromatograms (Fig. 1), the alcohol percentages for these appear proportionately higher in the anteiso-5:0 column of Table II. There is, however, every reason to suppose that the minor 2-methylbutyroyl esters all existed at undetectable levels or in positions covered by major components of other types.

The report of 3-methylbutyl (isoamyl) alcohol as a component of *T. gilli* mandible wax esters (7) prompted us to examine critically the short chain wax esters from *T. truncatus* jaw fat for such materials. Sixteen different 3-methylbutyl esters of C_{11}-C_{20} fatty acids were synthesized and cochromatographed with the *T. truncatus* short chain wax esters using GLC conditions especially developed for the purpose of detecting such compounds (10). All tests for 3-methylbutyl wax esters in *T. truncatus* jaw fat were negative. If such compounds did exist in the sample examined, the amount present must have been appreciably less than 1% of the short chain wax esters.

Substantial amounts of isovaleric acid have been found in the head fats of all members of the Odontocete families Delphinidae, Phocoenidae, and Monodontidae examined to date (2-9, 18-24). The simultaneous occurrence of

lesser amounts of isobutyric and 2-methylbutyric acids is by no means unique with *T. truncatus*, however. Isobutyric acid has been reported in *Delphinus delphis ponticus* (18), *Stenella attenuata* (21), and *Phocoenoides dalli* (19) head fats, while 2-methylbutyric acid was present in *D. delphis ponticus* (18) and *Globicephala melaena* (6). The parallel occurrence of isovaleroyl-CoA, isobutyroyl-CoA, and 2-methylbutyroyl-CoA during leucine, valine, and isoleucine metabolism respectively (25,26) apparently can produce esters of all three of the short chain acids in some animals of the Delphinidae and Phocoenidae. The phenomenon is not universal, however, since detailed GLC analyses have not revealed significant amounts of isobutyric acid in *Sotalia fluviatilis* (8) or *Delphinapterus leucas* (9).

The level of isobutyric acid found here in *T. truncatus* jaw fat is by far the highest value reported to date for any Odontocete head fat. Moreover, it represents the first known occurrence of a major amount of isobutyroyl wax ester in the fats of these animals.

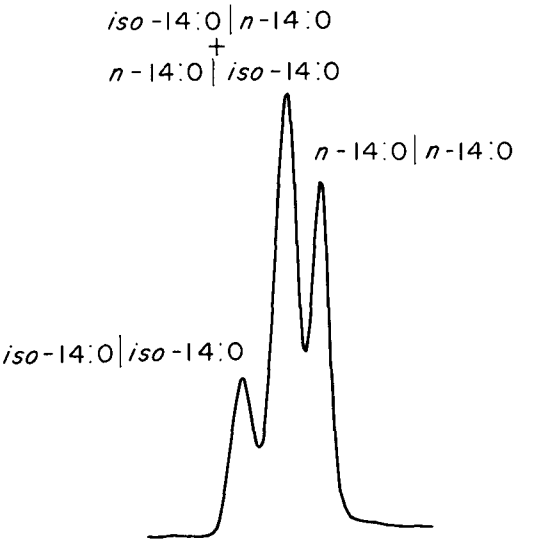

FIG. 3. Separation of a mixture of 12-methyltridecyl 12-methyltridecanoate (iso-14:0|iso-14:0), 12-methyltridecyl myristate (iso-14:0|n-14:0), tetradecyl 12-methyltridecanoate (n-14:0|iso-14:0), and tetradecyl myristate (n-14:0|n-14:0) into three peaks on a short, packed JXR column (0.53 m x 2.4 mm inside diameter stainless steel column packed with 3.0% JXR silicone on 100/120 mesh Gas Chrom Q). Operating conditions: column temperature programed 145 → 300 C at 3.3°/min, 100 ml/min He carrier gas, on-column injection at 325 C.

Long Chain Wax Esters

GLC analysis of the fatty acid methyl esters and the fatty alcohol acetates derived from the long chain wax esters are reported in Table II. The composition of these alcohols closely resembled those found in the short chain wax esters, indicating a common pool for all wax ester alcohols and a lack of alcohol specificity in the acyl transferase(s) which synthesize wax esters. The 2:1 proportion of 18:1ω9: 18:1ω7 differs from that of the acids (about 5:1) but is the same as that for the jaw wax ester fatty alcohols of the Amazon dolphin *Inia geoffrensis* (11).

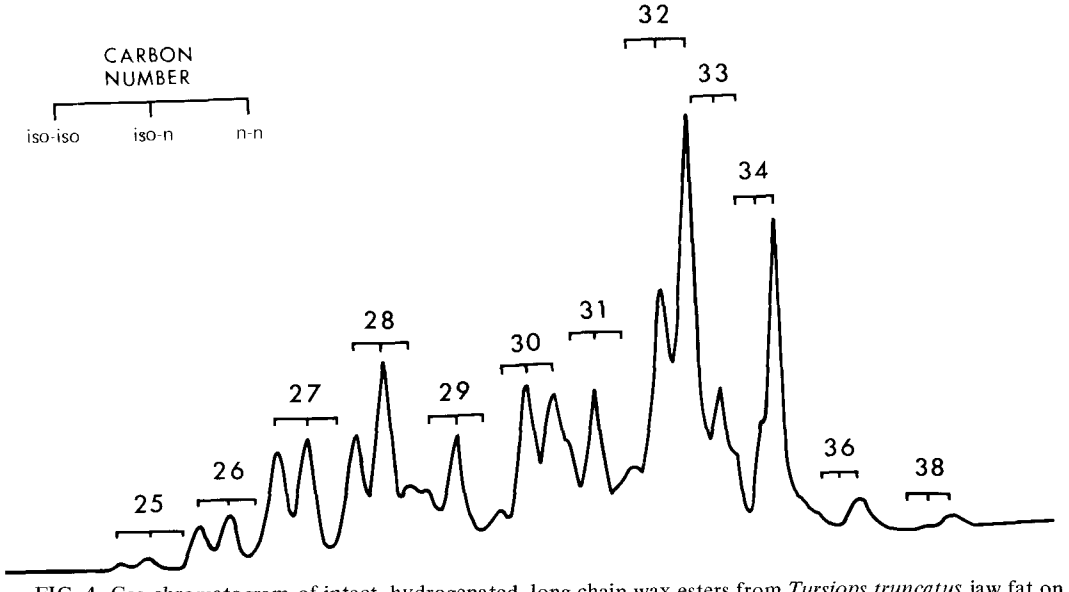

FIG. 4. Gas chromatogram of intact, hydrogenated, long chain wax esters from *Tursiops truncatus* jaw fat on a short, packed JXR column. Operating conditions: same as Figure 3 except column temperature programed 150 → 330 C at 4.6°/min.

TABLE III

Composition of Intact Long Chain Wax Esters from *Tursiops truncatus* Jaw Fat

Carbon number	Predominant structure	Mole % Found	Mole % Calculated[a]	Carbon number	Predominant structure	Mole % Found	Mole % Calculated
25	iso/iso	trace	0.1	30	n/n[c]	6.6	8.7
25	iso/n[b]	0.6	0.2	31	iso/iso		
26	iso/iso	1.1	0.4	31	iso/n	5.9	6.5
26	iso/n	1.4	1.1	32	iso/iso	1.3	5.5
27	iso/iso	4.7	1.6	32	iso/n	11.8	17.0
27	iso/n	4.2	1.1	32	n/n[c]	19.1	16.5
28	iso/iso	4.8	0.7	33	iso/iso		
28	iso/n	9.9	1.8	33	iso/n[c]	3.2	8.6
28	n/n	3.2	2.3	33	n/n		
29	iso/iso			34	iso/n	8.5	10.9
29	iso/n	4.2	2.1	34	n/n[c]		
30	iso/iso	0.9	1.6	36	n/n	0.6	4.0
30	iso/n	7.8	5.7	38	n/n	0.2	1.4
				Other		---	2.2

[a]Calculated assuming a random esterification of fatty alcohols with fatty acids. Values given combine n/n-C_x and iso/iso-C_{x+1} into one peak.

[b]The term iso/n here refers to the combined iso/n and n/iso wax esters, since these two isomers are inseparable in the gas liquid chromatographic analysis used. Iso actually includes both iso and anteiso chains, but very few of the latter are present (Table II).

[c]Major component in this combined peak.

On the other hand, the fatty acids of the long chain wax esters were quite distinctive from the alcohols. Numerous C_{10}-C_{14} and C_{18}-C_{22} species were found only among the acids. Palmitate was the most prominent component. The mixture contained 73.8% saturated acids, including 24.2% branched (mostly iso), and 25.7% monoene acids. Almost no polyunsaturated acids (only 0.5%) were present, implying that the wax ester long chain acids are not of dietary origin but are probably synthesized directly in the lower mandible fat body. The high level of iso-chains in both the acids (22.0%) and alcohols (46.8%) reflects the availability of iso-4:0 and iso-5:0 substrates for de novo biosynthesis of long chain fatty acids (27).

GLC of the intact, hydrogenated, long chain wax esters on a JXR silicone column obviously must produce a complex pattern of peaks, because of the high level of branched chain acids and alcohols present (Table II). To aid in peak identification, the precise separation of a known mixture of iso-14:0/iso-14:0, iso-14:0/n-14:0, n-14:0/iso-14:0, and n-14:0/n-14:0 under similar GLC operating conditions was investigated. Three distinct peaks were partially resolved (Fig. 3) with the iso/iso combination eluting first, the iso/n and n/iso structures forming an unresolved second peak, and the n/n unbranched compound eluting last, as predicted from basic GLC structure-retention time relationships (16). Based upon the separation of this model mixture and upon cochromatography runs with appropriate standards, all of the peaks in the complex chromatogram of long chain wax esters can be assigned (Fig. 4) and quantitated (Table III). Partial resolution of peaks makes the accuracy here lower than is usual for GLC of long chain wax esters, but an adequate quantitation can be obtained. In a few cases, two species of wax ester (such as C_{30} n/n and C_{31} iso/iso) eluted almost simultaneously and could not be quantitated individually; however, examination of the chromatogram and knowledge of the fatty acids and alcohols present allowed the major components to be identified.

Our results show that the long chain wax esters are ∿16% iso/iso, ∿47% iso/n and ∿37% n/n structures. This is close to the 12% iso/iso, 49% iso/n, 39% n/n composition calculated for a random esterification of the fatty acids with the fatty alcohols. However, comparison of experimental and random distribution values for both isomeric and carbon number separations (Table III) shows poor agreement. More C_{27}-C_{29} and less C_{32}-C_{36} species are present than could be accounted for by a random esterification pattern.

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REFERENCES

1. Gill, A.H., and C.M. Tucker, Oil Fat Ind. 7:101 (1930).
2. Litchfield, C., and A.J. Greenberg, Comp. Biochem. Physiol. In press.
3. Litchfield, C., and A.T. Greenberg, Paper presented at the 46th Fall Meeting, AOCS, Ottawa, Canada, September 1972, Abstract 27.
4. Tsujimoto, M., and H. Koyanagi, J. Soc. Chem. Ind. Japan 40 (Suppl. binding):272B (1937).
5. Wedmid, G., C. Litchfield, R.G. Ackman and J.C. Sipos, JAOCS 48:332A (1971).
6. Blomberg, J., Program of the XI Congress of the International Society for Fat Research, Göteborg, Sweden, 1972, Abstract 223.
7. Varanasi, U., and D.C. Malins, Biochemistry 9:3629 (1970).
8. Litchfield, C., J. Kinneman, R.G. Ackman and C.A. Eaton, JAOCS 48:91A (1971).
9. Litchfield, C., R.G. Ackman, J.C. Sipos and C.A. Eaton. Lipids 6:674 (1971).
10. Litchfield, C., and R.G. Ackman, J. Chromatogr. 75:137 (1973).
11. Ackman, R.G., C.A. Eaton and C. Litchfield, Lipids 6:69 (1971).
12. Norris, K.S., in "Evolution and Environment", Yale University Press, New Haven, Edited by E.T. Drake, 1968, pp. 297-324.
13. Litchfield, C., "Analysis of Triglycerides," Academic Press, New York, 1972, p. 38.
14. Mattson, F.H., R.A. Volpenhein and J.B. Martin, J. Lipid Res. 5:374 (1964).
15. Freeman, N.K., Ibid. 5:236 (1964).
16. Ackman, R.G., J. Chromatogr. Sci. 10:243 (1972).
17. Ackman, R.G., in "Progress in the Chemistry of Fats and Other Lipids," Edited by R.T. Holman, Pergamon Press, Oxford, 1972, p. 165.
18. Schlenk, W., Jr., JAOCS 42:945 (1965).
19. Nakamura, Y., and I. Tsujino, J. Agr. Chem. Soc. Japan 27:642 (1952).
20. Takaoka, M., and I. Tsujino, Bull. Jap. Soc. Sci. Fish. 18:58 (1952).
21. Morii, H., and R. Kanazu, Ibid. 38:599 (1972).
22. Varanasi, U., and D.C. Malins, Science 176:926 (1972).
23. Varanasi, U., and D.C. Malins, Biochemistry 9:4576 (1970).
24. Varanasi, U., and D.C. Malins, Biochim. Biophys. Acta 231:415 (1971).
25. Lehninger, A.L., "Biochemistry," Worth, New York, 1970, pp. 441-448.
26. Malins, D.C., P.A. Robisch and U. Varanasi, Biochem. Biophys. Res. Commun. 48:314 (1972).
27. Horning, M.G., D.B. Martin, A. Karmen and P.R. Vagelos, J. Biol. Chem. 236:669 (1961).

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Incorporation of Acetate into Fatty Acids and Complex Lipids of Soybean Cotyledon Slices under Aerobic and Anaerobic Conditions

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ABSTRACT

Soybean slices incubated with [1-^{14}C] acetate in the presence of air synthesized fatty acids to a greater extent than did slices in the absence of air. The proportion of radioactive fatty acids incorporated into the neutral lipid was ca. 35% in the presence or absence of air. However, both the proportion and the absolute amount of radioactive fatty acids in phospholipids were greater in the presence of air. This difference was particularly great in phosphatidic acid and in a minor uncharacterized phospholipid component. Significant incorporation of acetate into monoenoic acids was observed in these two lipids and in phosphatidyl choline. The latter also showed an accumulation of newly synthesized polyenoic acids when air was present. Stearic acid synthesis was greater under aerobic than under anaerobic conditions. The present results support the concept that a relationship exists between the synthesis of unsaturated fatty acids and their incorporation into phospholipids in plants.

INTRODUCTION

Synthesis of oleic acid from acetate or from saturated acids of less than 16 carbon atoms has been demonstrated in preparations of avocado mesocarp (1), leaves (2), and seeds (3,4). Exclusion of oxygen prevented the synthesis of oleic acid and caused the accumulation of palmitic and stearic acids. Pulse labeling in several higher plant systems generally has failed to provide support for desaturation of palmitic and stearic acids (5). Except for some reports on experiments with soybeans (5,6) and safflower (Jaworski and Stumpf, Abstract 2360, 57th Annual Meeting of the Federation of American Societies for Experimental Biology, April 1973), exogenous palmitic and stearic acids have not been shown to serve as direct precursors of oleic acid in higher plants. Endogenous stearic acid, synthesized anaerobically by leaves from acetate, was shown to be at least partly converted to oleic acid after exposure of the leaves to air (7,8). This raises the question whether a separate plant pathway to oleic acid exists or whether a desaturase exists having a requirement for a substrate form not exchangeable with exogenous stearic acid. Aerobic desaturation of endogenous and exogenous oleic acid to linoleic and α-linolenic acids has been reported to occur in leaves (9) and seeds (5,10) of higher plants. Anaerobic synthesis of α-linolenic acid by probable elongation of the corresponding hexadecatrienoic acid recently has been reported in spinach chloroplasts as well (11).

A close association between the formation of unsaturated fatty acids and their incorporation into polar lipids has been noted frequently (5), and recent reports implicate phosphatidyl choline as the major recipient of newly formed unsaturated fatty acids in *Chlorella vulgaris* (12), flax seeds (13), aged potato tubers (14), *Candida lipolytica* (15), and *Torulopsis utilis* (16). Enzymic modification of the carbon chain of a phospholipid-bound fatty acid in the synthesis of a cyclic fatty acid was demonstrated some time ago (17). Although recent reports (18-20) indicate that desaturation occurs in the chain of an acid bound as a thioester, rather than as an oxygen ester, this does not preclude the participation of phospholipids in some other way, perhaps as transfer agents or recipients.

In the present study, soybean slices were incubated with acetate in the presence or absence of air, and the phospholipid components were examined to determine whether any of them appear to be related specifically to the newly formed unsaturated fatty acids in the soybean.

MATERIALS AND METHODS

Randomized 1-2 mm slices of Chippewa 64 soybeans, harvested ca. 40 days after flowering, were incubated with sodium [1-^{14}C] acetate (New England Nuclear Corporation, Boston, Mass.) in buffer solution at 25 C with moderate shaking for 3-hr time periods. The buffer solution consisted of 0.1 M potassium phosphate 5 x 10^{-4} M in $MnSO_4 \cdot H_2O$ at pH 5.7 and contained 1 ppm biotin. Incubations were carried out in 25 ml Erlenmeyer flasks using 5.0 g of slices in 10 ml of buffer solution. Samples

to be treated anaerobically were sealed with serum stoppers, wrapped with aluminum foil and flushed with nitrogen for 45 min prior to the addition of acetate. Experiments commenced with the injection of 1.0 ml of slightly basic aqueous acetate (50 μCi). At the end of 3 hr, slices incubated aerobically were washed with fresh buffer solution and extracted. One-half of the anaerobically treated slices were washed, transferred to 10 ml of fresh buffer solution and allowed to shake in the presence of air for an additional 30 min before extraction. The other half of the anaerobically treated slices were washed under a layer of toluene and were extracted immediately.

Lipids were extracted from washed slices of each sample by grinding for 3 min at 45,000 RPM in 100 ml of boiling chloroform-methanol (2:1) in a VirTis model 45 homogenizer. After suction filtration through Whatman No. 1 filter paper, the resulting mat was rehomogenized twice in 100 ml portions of chloroform-methanol (1:1). The combined filtrates were concentrated in a rotary evaporator, and the lipid residues were redissolved in chloroform, filtered through glass wool, and made up to 10 ml volumes. Aliquots from these were used for subsequent analyses.

Preliminary fractionation of crude lipids was performed by subjecting 3 ml aliquots to column chromatography on 10 g of 60/100 mesh HCl-treated Florisil (Fisher Scientific Co., Fair Lawn, N.J.) (21) by the method of Rouser, et al. (22), as modified by Singh and Privett (23). The eluant was monitored by thin layer chromatography (TLC) on Silica Gel G (Brinkmann Instruments, Inc., Westbury, N.Y.) in petroleum ether (bp 30-60 C)-diethyl ether-acetic acid (70:30:2). Fraction I eluted by 200 ml of chloroform contained the neutral lipids. Fraction II eluted by 140 ml of chloroform-acetone (1:1), followed by 600 ml of acetone, contained the glycolipids and traces of phospholipids. Fraction III eluted by 200 ml of methanol contained the phospholipids. Each fraction was evaporated to a residue and made to 1 ml in chloroform. Phospholipids and glycolipids were identified on TLC plates by use of molybdate (24) and α-naphthol (25) reagents, respectively, and by comparison with authentic standard lipids. All standard lipids used in the study were purchased from the Lipids Preparation Laboratory of The Hormel Institute, Austin, Minn.

Further fractionation of phospholipid was performed in two ways. For determining the distribution of radioactivity among phospholipids, aliquots of Fraction III were subjected to two-dimensional TLC on sheets of instant TLC-silicic acid (Gelman Instrument Co., Ann Arbor, Mich.) using first chloroform-methanol-7N NH_4OH (65:30:4) and then chloroform-acetic acid-methanol-water (170:25:25:6) as solvents (26). Spots were made visible by spraying the sheets with 0.2% 2,7-dichlorofluorescein in ethanol and viewing under UV light and were cut from the sheets and counted by liquid scintillation spectrometry. For preparative purposes, aliquots of Fraction III were separated by TLC on Silica Gel H, using the above ammonia system and successive developments to 1/2, 3/4, and the total height of the plates with intermediate drying under nitrogen. Phospholipids were eluted from the silica gel with 10 volumes of methanol-chloroform-water (5:3:2) and were recovered by evaporation of the pooled lower phases after extractions with equal volumes of chloroform (once) and chloroform-methanol (85:15) (twice) (27).

Methyl esters were prepared from aliquots of crude lipid and of individual lipids by treatment with 10% sulfuric acid in methanol in sealed ampules at 100 C. Solvents were removed from aliquots prior to treatment. Samples on silica gel scrapings from TLC plates or sections cut from ITLC sheets were treated without prior extraction. Methyl esters extracted from the reaction mixture were purified by TLC on Silica Gel G in petroleum ether-diethyl-ether-acetic acid (90:10:1) prior to subsequent analysis.

Methyl esters were separated by two methods prior to measurement of radioactivity. Aliquots were separated by degree of unsaturation by argentation chromatography, according to the method of Graff et al. (28). Other aliquots were fractionated by gas liquid chromatography (GLC) on an 8 ft x 1/4 in. column packed with 15% EGSS-X on Gas Chrom P, 100/120 mesh (Applied Science Laboratories, Inc., State College, Pa.). Gas chromatography was performed in a gas chromatograph (600 series Research Specialties), equipped with a β-ionization detector, at 175 C with a flow of 60 ml/min of argon.

Radioactivities were measured in a Tri-Carb 314 EX liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.). Samples were dissolved in 15 ml of a scintillation solution prepared from 5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (Packard Instrument Co.) per liter of distilled reagent grade toluene. Lipid samples were dissolved in the scintillation solution after removal of other solvents. Sections cut from ITLC sheets first were eluted with 1 ml of methanol before addition of the scintillation solution to the vial. Inclusion of the ITLC

TABLE I

Incorporation of Sodium [1-^{14}C] Acetate into Soybean Cotyledon Lipid and Distribution between Neutral, Glyco-, and Phospholipid Fractions[a]

Incubation	Lipid incorporation per cent	Per cent distribution of ^{14}C		
		Neutral	Glycolipid	Phospholipid
Anaerobic	6.5	34	23	43
Anaerobic – aerobic	9.0	31	17	52
Aerobic	10.7	34	6	60

[a]Conditions of incubation and lipid fractionation in text.

section and the methanol was determined to cause less than 5% loss due to quenching.

N-palmitoyl phosphatidyl ethanolamine was prepared essentially according to Dawson et al. (29). Phosphatidyl ethanolamine (PE) (90 mg) was dissolved in 35 ml of washed and dried chloroform, plus 5 ml of pyridine. A solution of 200 mg of palmitoyl chloride in 25 ml of chloroform was added slowly and the reaction mixture was stirred at 40 C for half hr, after which time TLC monitoring indicated that nearly all PE had disappeared. Excess palmitate was removed then by careful washing with dilute bicarbonate, and the crude product was recovered by evaporation of the chloroform. The product was purified by TLC on Silica Gel G in chloroform-methanol-7N NH_4OH (70:30:5). The purified product exhibited the same R_f value reported by Dawson et al., and its IR spectrum was identical to that reported by Bomstein (30). Samples of the initial PE and the product were interesterified, and GLC analysis of the esters showed that an average of one equivalent palmitate had been added to two equivalents of original esters.

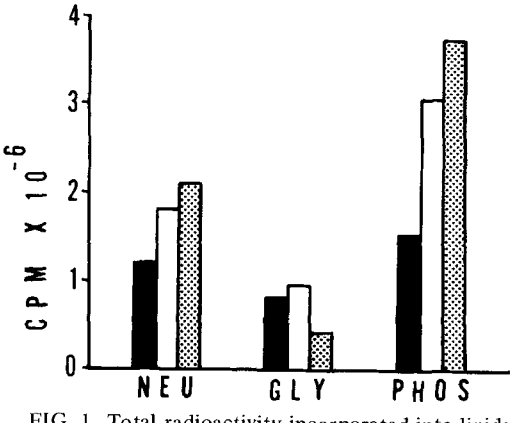

FIG. 1. Total radioactivity incorporated into lipids of soybean slices incubated with sodium [1-^{14}C] acetate. Conditions of incubation and fractionation in text. Solid bars = anaerobic treatment, open bars = anaerobic followed by aerobic treatment, shaded bars = aerobic treatment, NEU = neutral lipid, GLY = glycolipid, and PHOS = phospholipid.

RESULTS AND DISCUSSION

Incorporation of acetate into the total lipid of soybean cotyledon slices increased with exposure to air (see Table I). Slices incubated anaerobically, washed, and transferred to acetate-free medium under aerobic conditions showed a level of incorporation between those treated strictly anaerobically or aerobically. This sample generally gave values between those of the anaerobic and aerobic samples and, thus, permitted desaturation following de novo synthesis. Anaerobic-aerobic slices appear to have made use of absorbed acetate to a greater extent than did slices in the absence of air, but aerobically treated slices absorbed or utilized acetate still more effectively or rapidly than did the anaerobic-aerobic slices. In distribution of radioactivity, the neutral lipid was little affected by presence or absence of air, but glycolipid incorporated relatively less, and phospholipid relatively more acetate under aerobic conditions. When comparing total incorporation, it was seen that both neutral and phospholipid fractions incorporated more labeled acetate in the presence of air than in its absence (see Fig. 1). This trend was not found with the glycolipid fraction, perhaps because of analytical difficulties with the small samples. Under all three conditions of incubation, the greatest relative and absolute amount of radioactivity resided in the phospholipid, and access to air increased both the proportion and absolute amount. This supports our earlier findings that the newly synthesized fatty acids appear to be most rapidly incorporated into the phospholipid fraction and only later into the neutral lipid of the soybean (5). Since the neutral lipid represented ca. 90% of the total lipid, its specific activity was less by a factor of 10 than that of the phospholipid and; therefore, the neutral lipid was not investigated further in this study.

TABLE II

Incorporation of Sodium [1-^{14}C] Acetate into Total Phospholipids and Distribution between Individual Phospholipids[a]

Incubation	Phospholipid incorporation CPM x 10^{-3}	Per cent distribution of ^{14}C				
		PA	X	PC	PE	PS + PI
Anaerobic	1527	9	6	37	9	33
Anaerobic – aerobic	3052	20	10	24	7	28
Aerobic	3731	32	16	20	4	24

[a]PA = phosphatidic acid, X = unidentified minor phospholipid, PC = phosphatidyl choline, PE = phosphatidyl ethanolamine, PS = phosphatidyl serine, and PI = phosphatidyl inositol.

Individiual phospholipid classes were separated and analyzed to determine the effect of air on their content of radioactive fatty acids (see Table II). In the presence of air, relatively increased incorporation was particularly evident in phosphatidic acid (PA) and compound X, a very minor phospholipid to be described below, whereas phosphatidyl choline (PC), PE, and phosphatidyl serine + inositol (PS + PI) contained relatively less radioactivity. No other phospholipids were found to contain significant radioactivity.

In terms of absolute radioactivity in each fraction (Fig. 2), all of the phospholipids, with the possible exception of PE, increased in amount of ^{14}C when incubated aerobically, though increases in the PC and PS + PI fractions were less than the increase in total phospholipids. Absolute differences were six-fold or greater in the PA and X components. Singh and Privett (31) reported that soybean cotyledons treated with acetate for 15 min-24 hr incorporated radioactivity into the same lipids reported here and into phosphatidyl glycerol (PG) and N-acylphosphatidyl ethanolamine (N-acyl PE). In their study, PA decreased and N-acyl PE increased in proportion of radioactivity with time, whereas the value of PC rose and plateaued.

Since anaerobic treatment was used to inhibit the synthesis of unsaturated fatty acids, it was of prime interest to determine the comparative effects of the presence or absence of air on the composition of each phospholipid fraction. Distribution of radioactivity in the saturated and unsaturated fatty acids of each phospholipid (see Table III) shows that under anaerobic conditions, little radioactivity was in unsaturated fatty acids, whereas incubation in the presence of air resulted in very significant proportions in the monoenoic acids of PA, X, and PC and in the polyenoic acids of the latter. Determination of the total radioactivity in each acid (see Fig. 3) showed that both saturated and unsaturated acids contained generally greater radioactivity in the presence of air than in its absence and that the larger amount of

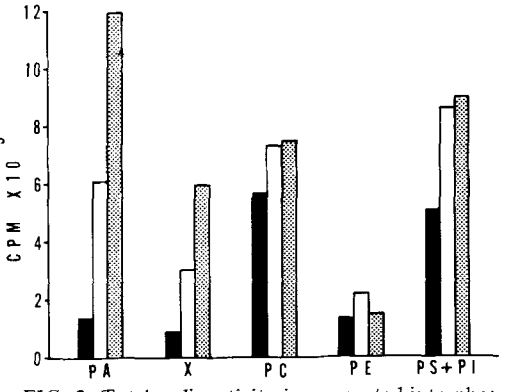

FIG. 2. Total radioactivity incorporated into phospholipids of soybean slices incubated with sodium [1-^{14}C] acetate. Solid bars = anaerobic treatment, open bars = anaerobic followed by aerobic treatment, shaded bars = aerobic treatment, PA = phosphatidic acid, X = uncharacterized phospholipid, PC = phosphatidyl choline, PE = phosphatidyl ethanolamine, PS = phosphatidyl serine and PI = phosphatidyl inositol.

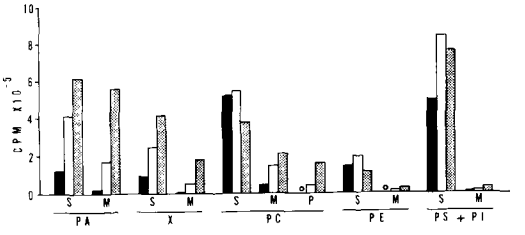

FIG. 3. Total radioactivity incorporated into phospholipid fatty acids of soybean slices incubated with sodium [1-^{14}C] acetate. Solid bars = anaerobic treatment, open bars = anaerobic followed by aerobic treatment, shaded bars = anaerobic treatment, PA = phosphatidic acid, X = uncharacterized phospholipid, PC = phosphatidyl choline, PE = phosphatidyl ethanolamine, PS = phosphatidyl serine, PI = phosphatidyl inositol, S = saturated, M = monoenoic, and P = polyenoic fatty acid methyl esters.

TABLE III

Incorporation of Sodium [1-^{14}C] Acetate into Individual Phospholipids and Distribution in Fatty Acids by Unsaturation

Phospholipid[a]	Incubation	Phospholipid incorporation CPM x 10^{-3}	Per cent distribution of ^{14}C			
			Saturated	Monoene	Diene	Triene
PA	Anaerobic	137	88	12	—	—
	Anaerobic – aerobic	610	68	28	3	1
	Aerobic	1194	52	47	1	—
X	Anaerobic	92	96	4	—	—
	Anaerobic – aerobic	305	81	17	2	—
	Aerobic	597	70	30	—	—
PC	Anaerobic	565	93	7	—	—
	Anaerobic – aerobic	732	75	20	4	1
	Aerobic	746	51	28	12	9
PE	Anaerobic	137	100	—	—	—
	Anaerobic – aerobic	214	90	5	5	—
	Aerobic	149	72	16	6	6
PS + PI	Anaerobic	504	99	1	—	—
	Anaerobic – aerobic	855	98	1	1	—
	Aerobic	895	85	11	3	—

[a]PA = phosphatidic acid, X = unidentified minor phospholipid, PC = phosphatidyl choline, PE = phosphatidyl ethanolamine, PS = phosphatidyl serine, and PI = phosphatidyl inositol.

activity in monoenes of slices incubated aerobically did not necessarily appear at the expense of the saturated acids. Only in the PC was an increase in unsaturated activity accompanied by a decrease in saturated acid activity. The PE and PS + PI fractions contained little newly formed monoenoic acid. Studies of oleic acid desaturation to linoleic acid in *Chlorella* (12), yeast (15,16), tubers (14), and seeds (20) have shown that the linoleic acid accumulates primarily in the PC fraction. However, no single phospholipid has, as yet, been implicated similarly as the major site of oleic acid accumulation, though phospholipids, as a fraction, previously have been shown to contain the bulk of newly synthesized oleic acid (5). Results in Table III and Figure 3 indicate that PC was the only phospholipid which contained a significant amount of radioactive linoleic acid, whereas oleic acid was found primarily in PA and compound X.

TABLE IV

Fatty Acid Compositions of Phospholipid Fractions of 40-Day Soybean Slices by Gas Liquid Chromatography

Phospholipid[a]	Per cent composition				
	16:0	18:0	18:1	18:2	18:3
PA	25	7	7	51	10
X	33	8	8	45	6
PC	27	8	8	52	5
PE	50	4	5	38	3
PS + PI	43	10	5	36	6

[a]PA = phosphatidic acid, X = unidentified minor phospholipid, PC = phosphatidyl choline, PE = phosphatidyl ethanolamine, PS = phosphatidyl serine, and PI = phosphatidyl inositol.

Neither per cent distribution nor total radioactivity in the fatty acids reflects dilution by fatty acids already contained by the phospholipids. Table IV presents the fatty acid composition of each fraction. All phospholipids were composed largely of saturated and dienoic acids. Therefore, incorporation of acetate into monoenoic acids would cause a relatively greater change in specific activity than the same amount of incorporation into saturated fatty acids. Table V provides comparison of ratios of radioactivity to composition (specific activities) for the saturated and monoenoic fractions of each phospholipid. Increases of up to five-fold for the saturated acids occurred during aerobic incubation, as compared with anaerobic; however, far greater increases in the specific activities of monoenoic acids were observed and this was especially evident in the PA and X phospholipids. GLC of saturated methyl esters, prepared from each phospholipid, showed that most of the radioactivity was present in 16- and

TABLE V

Ratios of Total Radioactivity to Per Cent Composition for Saturated and Monounsaturated Acids of Soybean Phospholipids after Anaerobic and Aerobic Incubation of Slices with Sodium [1-^{14}C] Acetate

	CPM x 10^{-3}/Per cent composition			
	Saturated		Monoenoic	
Phospholipid[a]	Anaerobic	Aerobic	Anaerobic	Aerobic
PA	3.8	19.4	2.3	80.2
X	2.2	10.2	0.5	22.4
PC	15.0	10.9	4.9	26.1
PE	2.5	2.0	0	4.8
PS + PI	9.4	14.4	1.0	19.7

[a]PA = phosphatidic acid, X = unidentified minor phospholipid, PC = phosphatidyl choline, PE = phosphatidyl ethanolamine, PS = phosphatidyl serine, and PI = phosphatidyl inositol.

18-carbon acids. In all phospholipids, except compound X, stearic acid contained a relatively greater, and palmitic acid a relatively smaller proportion of the radioactivity when air was present during the incubation. This was particularly evident in the PA fraction. It would appear that the presence of air favors chain elongation of palmitic acid, as well as being essential for desaturation to occur. This contrasts somewhat with the findings of James and coworkers (7,8) that, in leaves, anaerobic synthesis from acetate led to the formation of stearic acid, which then was desaturated to oleic on exposure of the leaves to air.

The phospholipid designated as compound X was found to be present in small amounts (<0.1% of total lipid) but was of much greater specific activity than the other phospholipids. It showed a far greater incorporation of radioactivity under aerobic conditions than under anaerobic conditions and it showed an equally dramatic difference in monoenoic radioactivity and specific activity. This component chromatographed with phospholipids on Florisil and gave a positive phosphomolybdic acid test on thin layer plates. In TLC, the compound behaved in a manner similar to that of the N-acyl PE reported by Singh and Privett. However, cochromatography with authentic N-acyl PE on ITLC-SA plates in two dimensions showed that radioactive compound X chromatographed very close to, but not coincident with, the standard. The same phospholipid prepared from young untreated soybeans did chromatograph coincident with the radioactive sample, but it failed to exhibit maxima for amine or amide in the IR spectrum.

Results of this study confirm and extend the previous observations of a relationship between synthesis of unsaturated fatty acids in plants and their occurrence as components of phospholipids. Specifically PA and compound X are identified as lipids in which newly formed oleic acid accumulates, and PC is identified as the major depository of linoleic acid. PA, and perhaps other phospholipids, appear similarly related to stearic acid. Although it is tempting to postulate desaturation of fatty acids while they are components of phospholipids, Stumpf (19,20) has presented evidence that the thioester, rather than the oxygen ester, is the substrate for desaturation in plants. This does not preclude possible participation of phospholipids in transport to sites of desaturase activity.

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REFERENCES

1. Mudd, J.B., and P.K. Stumpf, J. Biol. Chem. 236:2602 (1961).
2. James, A.T., Biochim. Biophys. Acta 70:9 (1963).
3. Hawke, J.C., and P.K. Stumpf, J. Biol. Chem. 240:4746 (1965).
4. Drennan, C.H., and D.T. Canvin, Biochim. Biophys. Acta 187:193 (1969).
5. Stearns, E.M., Jr., in "Progress in the Chemistry of Fats and Other Lipids," Vol. IX., edited by R.T. Holman, Pergamon Press, Oxford, England, 1970, pp. 453.
6. Inkpen, J.A., and F.W. Quackenbush, Lipids 4:539 (1969).
7. James, A.T., P. Harris, and J. Bezard, Eur. J. Biochem. 3:318 (1968).
8. Harris, R.V., A.T. James, and P. Harris, in "Biochemistry of Chloroplasts," Vol. II., edited by T.W. Goodwin, Academic Press, New York, 1967, p. 250.
9. Harris, R.V., and A.T. James, Biochim. Biophys. Acta 106:456 (1965).
10. McMahon, V., and P.K. Stumpf, Biochim. Biophys. Acta 84:359 (1964).
11. Jacobson, B.S., C.G. Kannangara and P.K. Stumpf, Biochem. Biophys. Res. Commun.

51:487 (1973); 52:1190 (1973).
12. Gurr, M.I., M.P. Robinson and A.T. James, Eur. J. Biochem. 9:70 (1969).
13. Dybing, C.D., and B.M. Craig, Lipids 5:422 (1970).
14. Ben Abdelkader, A., A. Cherif, C. Demandre and P. Mazliak, Eur. J. Biochem. 32:155 (1973).
15. Kates, M., and M. Paradis, Can. J. Biochem. 51:184 (1973).
16. Talamo, B., N. Chang and K. Bloch, J. Biol. Chem. 248:2738 (1973).
17. Law, J.H., Biochemistry 3:967 (1964).
18. Vijay, I.K., and P.K. Stumpf, J. Biol. Chem. 246:2910 (1971).
19. Kannangara, C.G., and P.K. Stumpf, Biochem. Biophys. Res. Commun. 44:1544 (1971).
20. Stumpf, P.K., Biochem. J. 128:3P (1972).
21. Carroll, K.K., JAOCS 40:413 (1963).
22. Rouser, G., G. Kritchevsky, G. Simon and G.J. Nelson, Lipids 2:37 (1967).
23. Singh, H., and O.S. Privett, Lipids 5:692 (1970).
24. Vaskovsky, V.E., and E.Y. Kostesky, J. Lipid Res. 9:396 (1968).
25. Siakotos, A.N., and G. Rouser, JAOCS 42:913 (1965).
26. Nichols, B.W., in "New Biochemical Separations," edited by A.T. James, D. VanNostrand Co., London, 1964, p. 332.
27. Schmid, H.H.O., and T. Takahashi, J. Lipid Res. 11:412 (1970).
28. Graff, G., Y.L. Marcel and R.T. Holman, J. Chromatogr. Sci. 7:298 (1969).
29. Dawson, R.M.C., N. Clarke and R.H. Quarles, Biochem. J. 114:265 (1969).
30. Bomstein, R.A., Biochem. Biophys. Res. Commun. 21:49 (1965).
31. Singh, H., and O.S. Privett, Biochim. Biophys. Acta 202:200 (1970).

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Fatty Acid Positional Distribution in Egg Yolk Triglycerides from Various Avian Species

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ABSTRACT

The fatty acid composition and distribution in egg yolk triglycerides and phosphatides from the turkey, duck, prairie chicken, bobwhite quail, Japanese quail, and inbred-hybrid and midget mutant hens were determined after all species had been fed diets of similar fat and fatty acid content for 90 days. Total yolk lipids were composed of ca. two-thirds neutral lipids and one-third polar lipids. The predominant fatty acids were palmitic and stearic. There were statistically significant differences in the myristic, palmitic, palmitoleic, linoleic, and linolenic acids in the yolk triglycerides and in the proportion of 16:1, 18:0, 18:2, arachidonic, docosanoic, docosahexaenoic, and tetracosanoic acids in the phosphatides among the various species. Linoleic acid predominantly was linked at the 2-position in the yolk triglycerides followed by the 20:4 acid. The 18:1 acid also was found preferentially at the 2-position. There was a low level of 18:2 in the yolk triglycerides and phosphatides from the duck and an especially high level of 20:4 acid in the phosphatides. The triglycerides in the species studied have essentially the same distribution of fatty acids in the 2-position. In all the species, the affinity for the fatty acids at the 2-position is in the following order: 18:2 = 20:4 > 18:1 = 18:3 > 18:0 = 16:1 > 14:0 > 16:0. Differences observed among the various genera did not appear to follow taxonomic boundaries. The duck has an efficient system for converting 18:2 into 20:4 by elongation and desaturation. The prairie chicken apparently has a high requirement for 18:2 but an inadequate system for its conversion into 20:4.

INTRODUCTION

Biochemical parameters have been introduced recently in an attempt to determine if classical systematics and biochemical differentiation are related. Comparative studies on the primary structure on cytochromes-C in various terrestrial, marine, and microbial species have been reported (1). The biosynthetic pathways of unsaturated fatty acids in microorganisms (2); interspecies differences in structure of endogenous triglycerides (3,4); the triglyceride composition of milk fat from seven mammalian species (5); the positional distribution of fatty acids in fish and marine mammal triglycerides (6); and the triglyceride composition of depot fat in various mammals, birds, fish, and amphibians (7) have been investigated from the comparative standpoint.

Several classifications have been proposed to systematize the thousands of bird species recognized to date. One of the recent ones (8) has subdivided the class Aves into 2 subclasses, Archaeornithes and Neornithes, and these into one and 33 orders, respectively. Each order has been separated further into various families, each of which, in turn, is composed of several genera, the latter comprising one or more species of birds.

Evidence has been presented (9,10) regarding the differences in fatty acid composition of egg yolks from domestic hens fed the same diet but belonging to different breeds of strains. Significant differences were found in the yolk content of 16:1, 18:0, 18:2, and 20:4 acids among the strains investigated. Similar studies (11) have disclosed further that existing differences in yolk fatty acid composition, due to strain of hens, are magnified as the fat content of the ration increases. The latter seems to

		NOMENCLATURE	
ORDER[1]	FAMILY	COMMON	SYSTEMATIC
Anseriformes	Anatidae	Duck[2]	*Anas Boschas*
		Laying hen[3]	*Gallus gallus, L.*
		Prairie chicken[4]	*Tympanuchus cupido attwaterii, Bendire*
Galliformes	Phasianidae	Japanese quail	*Coturnix coturnix japonica*
		Bobwhite quail	*Colinus virginianus*
	Meleagrididae	Turkey[5]	*Meleagris gallopavo*

FIG. 1. Systematic interrelationship among the avian species whose yolk lipids were compared. All orders listed belong in the class aves, subclass Neornithes.

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TABLE I

Model of Analysis of Variance

Source of variation	Degrees of freedom	Expected mean square
Total	13	
Between avian species	6	$s^2_{sr}+k_2+s^2_s$
Within avian species	1	$s^2_{sr}+k_1+s_r$
Error	6	s^2_{sr}

indicate that different breeds of hens metabolize the various fatty acids differently. Thus, it would not be unreasonable to assume that the positional distribution of fatty acids in egg yolk triglycerides may vary among different species of birds.

The purpose of the study reported herein was to determine the positional distribution of fatty acids in yolk triglycerides from the domestic turkey (*Meleagris gallopavo*), bobwhite quail (*Colinus virginianus*), Japanese quail (*Coturnix coturnix japonica*), Attwater prairie chicken (*Tympanuchus cupido attwaterii, Bendire*), domestic duck (*Anas boschas*), and the domestic hen (*Gallus gallus* L., normal inbred-hybrid and midget mutant). The systematic interrelationship of these avian species is diagramed in Figure 1.

MATERIALS AND METHODS

Experimental Procedure

Laying hens used in this study were normal inbred-hybrid and midget mutants. The turkey hens were the Beltsville Small White variety. The ducks were the Khaki Campbell strain.

A diet composed of 21.46% ground yellow corn, 42.93% ground sorghum grain, 20.86% soybean oil meal, 2.00% menhaden fish meal, 3.00% dehydrated alfalfa leaf meal, 6.00% oyster shell flour, 2.50% defluorinated rock phosphate, 0.25% sodium chloride, and 1.00% vitamin and trace mineral mix was fed to the chicken hens (inbred-hybrid and midget mutant), duck, Japanese quail, and bobwhite quail. The second diet was composed of 20.50% ground yellow corn, 41.15% ground sorghum grain, 23.60% soybean oil meal, 2.00% menhaden fish meal, 3.00% dehydrated alfalfa leaf meal, 6.00% oyster shell flour, 2.50% defluorinated rock phosphate, 0.25% sodium chloride, and 1.00% vitamin and trace mineral mix. It was fed to the turkeys and prairie chickens. The vitamin and trace mineral premix used in both diets supplied the following/kg diet: 9900 international units (IU) vitamin A palmitate, 4400 international chick units (ICU) vitamin D_3, 4.4 mg riboflavin, 13.2 mg d-calcium pantothenate, 55 mg niacin, 0.013 mg vitamin B_{12}, 33 IU vitamin E (α-tocopherol acetate), 2.2 mg menadione sodium bisulfite, 126 mg ethoxyquin, 22 mg zinc bacitracin, 81.9 mg ZnO, 180 mg $MnSO_4$, 1.1 g choline chloride, and 50 mg 3-nitro-4-hydroxyphenylarsonic acid. There was one exception: the vitamin premix added to the diet for the prairie chicken contained 16,500 IU vitamin A/kg. The first diet contained 3.3% crude fat and the second 3.1%, as analyzed (12). These diets were fed as indicated for 90 days. After which time, eggs from each genera were collected for egg yolk lipid studies.

Biochemical Procedures

The lipid material was extracted from duplicate egg yolk samples by the procedure of Folch, et al., (13) and fractionated by the silicic acid slurry method of Murty, et al., (14). Yolk triglycerides were isolated from the neutral lipid fraction by preparative thin layer chromatography (TLC), according to the method described by Privett, et al., (15). Yolk triglycerides were separated on the basis of the number of ethylenic linkages per triglyceride molecule, according to the argentation procedures of Culp, et al., (16) and Blank, et al., (17). Egg yolk triglycerides were subjected to pancreatic lipase hydrolysis, according to the method of Luddy, et al., (18) for triglycerides containing one or more double bonds and to the procedure of Barford, et al., (19) for triglycerides containing only saturated components. The fatty acid moieties from the 2-monoglycerides, which resulted from the enzymatic lipolysis of triglycerides, were analyzed by gas liquid chromatography (GLC) (20).

The fatty acid composition of the yolk polar lipids and diets also was determined by GLC (20). Methyl esters of the fatty acid moieties were prepared, according to the method of Feldman, et al., (21) and analyzed, as outlined by Saloma, et al., (20).

Data obtained from this study were analyzed statistically by the procedures of Snedecor

(22). The fatty acid composition and distribution of yolk triglycerides and the fatty acid composition of egg phosphatides were appraised by means shown in Table I. Significantly different means were separated through the use of Duncan's Multiple Range Test (23).

RESULTS AND DISCUSSION

The total ether extract of the two diets listed was 3.3 and 3.1% respectively. The fatty acid analysis of the diets (Table II) showed only slight differences in fatty acid composition. The major fatty acid components in the diets (Table II) were 16:0, 18:1, and 18:2. Diet two contained more of 16:0 acid and diet one contained more of 18:2 acid.

TABLE II

Fatty Acid Composition of Lipids in Experimental Diets (wt. %)

Component[a]	Laying hen ration	Turkey breeder ration
8:0-12:0	1.0	1.0
14:0	2.8	2.4
16:0	17.7	21.0
16:1	3.3	3.9
18:0	5.6	7.7
18:1	21.2	22.9
18:2	39.8	35.0
18:3	4.9	4.7
20:3	1.0	Tr[b]
22:1	2.7	0.5

[a]Chain length: number of double bonds.
[b]Tr = trace (<0.4%).

Egg Yolk Lipids

There was some variation in the total lipid content of the egg yolks from the selected avian species (Table III). The lowest value for total egg yolk lipids was that of the midget mutant, and the highest was that of the Japanese quail and normal laying hen. In all cases, the neutral and polar lipids comprised ca. two-thirds and one-third of the total lipids, respectively. As reported earlier, it has not been possible to modify the lipid content of egg yolks through dietary procedures (24-26). The fact that the proportions of neutral and polar lipids remain constant in various avian species suggests that there is a genetic control of yolk fat deposition which is impervious to dietary or environmental changes in birds. It is assumed that differences in the fatty acid composition of egg yolk lipids from the various species in this study were due to metabolism or absorption of each fatty acid, both of which might be traceable to genetic variation.

Fatty Acid Composition of Yolk Triglycerides

The major fatty acids found in the egg yolk triglycerides from the various avian species studied were 16:0, palmitoleic acid, stearic acid, 18:1, and 18:2 (Table IV). The 16:0 acid content of egg yolk lipids from the seven different birds studied varied from 23.5-30.3%. The highest level was found in the yolk lipids from the bobwhite quail and the lowest from that of the duck. Yolk lipids from the prairie chicken and turkey were significantly higher in the 16:1 acid content. These were followed by that of the Japanese and bobwhite quails with the lowest levels of this acid being bound in yolk lipids from the laying hen and duck. There was no significant difference in 18:0 acid content of yolk lipids from the seven birds studied. The yolk lipid content of 18:1 acid varied from 55.3 (duck) - 39.6% (turkey). The 18:1 acid content of the prairie chicken, bobwhite quail, and turkey was lower than that of the other species studied. There was a particularly low level of 18:2 acid found in the egg yolk lipids from the duck. This was followed, in turn, by the prairie chicken and Japanese quail. Possibly, the duck has a low requirement for linoleic acid or the diet used did not contain a sufficient level of this acid to meet the laying duck requirement. When there

TABLE III

Neutral Lipid, Polar Lipid, and Total Fat Content of Egg Yolks from Selected Avian Species Expressed as Percentage of Yolk Weight on a Wet Basis[a]

Species	Neutral lipids %	Polar lipids %	Total fat %
Japanese quail	67.5	32.5	33.0
Duck	63.5	36.5	31.0
Bobwhite quail	64.0	36.0	31.5
Prairie chicken	62.0	38.0	30.5
Turkey	64.0	36.0	29.0
Midget hen	64.0	36.0	27.0
Laying hen	61.5	38.5	33.0

[a]Means of duplicate samples.

TABLE IV

Fatty Acid Composition of Egg Yolk Triglycerides from Selected Avian Species (wt. %)[a]

Component[b]	Laying hen	Midget hen	Prairie chicken	Japanese quail	Bobwhite quail	Turkey	Duck
14:0[c]	0.5[2]	0.6[4]	0.8[1]	0.8[1]	0.8[1]	0.7[4]	0.6[4]
16:0	24.8[5]	25.6[5]	29.4[1]	25.7[5]	30.3[1]	27.9[4]	23.5[3]
16:1	4.6[3]	5.6[3]	10.2[1]	7.3[2]	7.3[2]	10.5[1]	4.3[3]
18:0	5.3	8.1	4.9	6.9	5.4	4.8	5.9
18:1	53.4[4]	47.1[6]	44.9[5]	48.1[6]	41.3[3]	39.6[3]	55.3[1]
18:2	10.2[1]	11.2[1]	8.5[4]	9.3[1]	12.5[1]	14.2[1]	6.9[2]
18:3	0.5[2]	1.0[4]	0.8[2]	0.9[4]	1.1[4]	1.7[1]	1.3[4]
% SFA[d]	30.7	34.9	35.4	33.9	37.1	33.8	31.0
% MUFA	58.0[4]	52.7[4]	55.0[4]	55.4[9]	48.6[2]	50.0[2]	59.8[1]
% PUFA	11.0	12.5	9.4	10.5	14.3	16.2	9.4

[a]Means of duplicate samples.

[b]Chain length: number of double bonds.

[c]Within each component, means bearing different superscripts are significantly different ($P < .05$).

[d]S = saturated; MU = monounsaturated; PU = polyunsaturated; FA = fatty acid; Tr = trace (<0.4%).

is a high level of the 18:1 acid in the egg yolk lipids, there is a correspondingly decreased level of the 18:2 acid.

Fatty Acid Composition of the 2-Position of Egg Yolk Triglycerides

There were no great differences in the linkage of the 16:0 acid at position 2 of the triglycerides which could be traceable to species (Table V). There was a significant difference between some of the species, as for example the prairie chicken, but there was an overlapping of values in the various species. The prairie chicken deposited the highest level of 16:1 acid at the 2-position in the egg yolk triglycerides followed by the turkey. The values for the 18:0 acid linked at the 2-position were rather low and without significance between species. The majority of the acids linked at the 2-position were 18:1 and 18:2. The bobwhite quail and turkey had the lowest quantity of 18:1 acid linked at the 2-position in the egg yolk triglycerides, whereas the Japanese quail had the highest value. The duck had by far the lowest quantity of 18:2 acid linked at the 2-position in egg yolk triglycerides, followed in order by prairie chicken and Japanese quail. The highest values of 18:2 acid linked at the 2-position were found in the egg yolk triglycerides from the laying hen, bobwhite quail, and turkey. The figures for the quantity of 18:2 acid linked at

TABLE V

Fatty Acid Composition of Position 2 in Egg Yolk Triglycerides from Selected Avian Species (wt. %)[a]

Component[b]	Laying hen	Midget hen	Prairie chicken	Japanese quail	Bobwhite quail	Turkey	Duck
14:0[c]	0.4	0.4	Tr	Tr	Tr	Tr	Tr
16:0	4.5[2]	6.7[4]	8.1[1]	4.0[2]	6.6[4]	6.8[4]	6.0[4]
16:1	2.9[3]	3.5[3]	8.0[1]	3.8[3]	4.0[5]	6.3[4]	2.8[3]
18:0	3.6	5.8	3.2	3.1	2.9	3.2	4.3
18:1	57.9[5]	57.7[5]	59.8[6]	68.4[4]	54.9[3]	51.6[3]	69.8[1]
18:2	29.1[1]	23.0[4]	17.7[5]	18.6[6]	27.6[4]	29.1[1]	12.2[3]
18:3	0.7	0.8	0.8	0.7	1.1	1.5	1.3
% SFA[d]	9.2	14.1	12.3	8.0	11.0	11.1	11.9
% MUFA	60.8[5]	61.6[5]	68.9[4]	72.2[1]	59.2[5]	57.9[3]	73.3[1]
% PUFA	30.2[1]	24.4[6]	18.8[3]	19.8[5]	29.7[4]	31.1[1]	15.0[3]

[a]Obtained from analysis of 2-monoglycerides resulting from pancreatic lipase hydrolysis of duplicate, intact triglyceride samples.

[b]Chain length: number of double bonds.

[c]Within each component, means bearing different superscripts are significantly different ($P < .05$).

[d]S = saturated; MU = monounsaturated; PU = polyunsaturated; FA = fatty acids; Tr = trace (<0.4%).

TABLE VI

Allotment of Individual Fatty Acids at 2-Position in Egg Yolk Triglycerides from Selected Avian Species[a]

Component[b]	Laying hen	Midget hen	Prairie chicken	Japanese quail	Bobwhite quail	Turkey	Duck
Percentage at 2-position							
16:0	6	9	9	5	5	8	9
16:1	21	21	26	18	18	20	22
18:0	23	24	22	15	18	22	24
18:1	36	41	44	47	44	43	42
18:2	95	69	69	67	74	68	59

[a]These data were calculated from the fatty acid composition of corresponding triglycerides and 2-monoglycerides.

[b]Chain length: number of double bonds.

the 2-position in egg yolk triglycerides follow closely the values for this acid found in the fatty acid composition of the egg yolk triglycerides (Table IV). The 18:2 acid linked at the 2-position was particularly low in case of duck yolk triglycerides, which may mean that this bird has a low requirement for linoleic acid or that the diet did not supply a sufficient quantity to meet the requirement.

Allotment of Individual Fatty Acids at the 2-Position

The percentage of 16:0 acid linked at the 2-position was low and varied from 5-9% (Table VI). There was a decided increase in the percentage of 16:1 acid linked at the 2-position with no great difference between species. With the exception of the Japanese and bobwhite quails, which had lower percentages of 18:0 acid linked at the 2-position than the other species, there was little difference remaining in the five other birds. A still further increase in the percentage of 18:1 acid was noted as being linked at the 2-position with the lowest value for the laying hen (36%) and no difference in the remaining six birds. The largest percentage of any fatty acid was that of 18:2 linked at the 2-position for all species studied, the percentages ranged from 59% in the case of the duck to 95% for the laying hen. With 18:3 acid there was a rather wide variation from 27% (midget mutant) to 47% (laying hen). Other values were intermediate.

If 33% fatty acid is in the 2-position of the triglycerides, then there is random distribution.

TABLE VII

Fatty Acid Composition of Polar Lipids in Egg Yolks from Selected Avian Species (wt. %)[a]

Component[b]	Laying hen	Midget hen	Prairie chicken	Japanese quail	Bobwhite quail	Turkey	Duck
14:0[c]	Tr	Tr	Tr	Tr	Tr	Tr	0.4
16:0	25.3	28.1	26.8	24.7	29.7	24.7	29.9
16:1	2.1^{2}	2.3^{2}	4.1^{1}	2.7^{2}	2.5^{2}	4.3^{1}	2.8^{2}
18:0	16.6^{1}	16.1^{6}	17.4^{1}	18.1^{1}	13.7^{2}	17.3^{1}	10.8^{3}
18:1	27.2	27.8	26.0	26.4	28.4	27.9	28.2
18:2	15.0^{2}	12.0^{3}	17.8^{1}	14.1^{2}	13.7^{7}	14.0^{2}	6.8^{4}
18:3	0.4	0.5	Tr	Tr	Tr	0.5	0.4
20:3	Tr	—	0.7	0.4	—	0.5	1.1
20:4	8.0^{2}	6.9^{7}	3.6^{5}	7.1^{2}	5.7^{8}	4.7^{9}	11.6^{1}
22:0	0.8^{2}	0.8^{2}	1.0^{2}	0.6^{2}	0.7^{2}	0.6^{2}	1.5^{1}
22:6	2.9^{1}	2.3^{1}	0.7^{2}	3.3^{1}	3.1^{1}	2.8^{1}	2.4^{1}
24:0	0.6	0.6	0.4	0.4	—	0.4	0.5
24:1	1.4^{7}	1.9^{2}	0.4^{4}	0.9^{8}	1.0^{3}	1.3^{3}	2.7^{1}
% SFA[d]	43.2	46.4	46.3	44.3	45.0	43.6	43.4
% MUFA	30.6^{3}	32.0^{2}	30.4^{3}	29.9^{3}	31.8^{2}	33.5^{1}	33.7^{1}
% PUFA	26.2^{1}	21.7^{2}	23.2^{2}	25.7^{1}	23.2^{2}	22.9^{2}	22.9

[a]Mean of duplicate samples.

[b]Chain length: number of double bonds.

[c]Within each component, means bearing different superscripts are significantly different ($P < .05$).

[d]S = saturated; MU = monounsaturated; PU = polyunsaturated; FA = fatty acids; Tr = trace (< 0.4%).

If less than 33% fatty acid is in the 2-position, this means that the fatty acid predominates in the 1- and 3-positions. If more than 33% fatty acid is in the 2-position then this acid predominates in the 2-position. The triglycerides in the species studied have essentially the same distribution of fatty acid in the 2-position. In all of the species, the affinity for fatty acid at the 2-position is in the following order: 18:2 = 20:4 > 18:1 = 18:3 > 18:0 = 16:1 > 14:0 > 16:0.

Fatty Acid Composition of Polar Lipids

There was little difference in 16:0 acid content of the polar lipids in egg yolks from the seven birds studied. Values obtained were within a narrow range (24.7-29.9), with no significant difference between species (Table VII). The 16:1 acid content of polar lipids ranged from 2.1-4.3% with the prairie chicken and turkey having the highest values, which were significantly different from that of the other species studied. The 18:0 acid content of the egg yolk polar lipids ranged from 10.8-17.4%. The 18:0 acid content of egg yolk polar lipids from the laying hen, prairie chicken, Japanese quail, and turkey was significantly higher than that of the other birds. There was no significant difference between the 18:1 acid content of the egg yolk polar lipids from the various species. The duck had a significantly low level of 18:2 acid in the egg yolk polar lipids, and the prairie chicken had the highest value. The 18:2 acid content of the egg yolk polar lipids from the laying hen was significantly higher than that of the midget mutant. The Japanese and bobwhite quails and the turkey had similar values. It is possible that the prairie chicken may have a high requirement for linoleic acid and the duck an especially low requirement. The docosahexaenoic acid (22:6) content of the egg yolk polar lipids was decreased significantly in the prairie chicken, with little difference existing in docosahexaenoic acid (22:6) content of egg yolk polar lipids from other species. Measurable amounts of 24:1 acid are reported, with the highest value being found in the egg yolk polar lipids of the duck, which was significantly higher than that of other species.

The egg yolk phosphatides from the duck contained 1.1% of the eicosatrienoic acid. This acid has been found to appear in the yolk lipids of chicken hens fed diets deficient in linoleic acid (27,28). The possibility of a suboptimum 18:2 acid intake by the duck might be questioned, since it has been shown that 18:2 is the precursor of 20:4 and docosapentaenoic acids (22:5) in the laying hen (29). The diets fed to the experimental birds contained no detectable arachidonic acid; all yolks analyzed were shown to contain varying proportions of 20:4 acid. Therefore, it is apparent that all species studied were capable of synthesizing arachidonic acid from the exogenous precursors available. The duck possesses the most efficient system for converting linoleic to arachidonic acid by elongation and desaturation. This hypothesis also would explain the observed decrease in 18:0 acid content of the duck egg phosphatides associated with the conversion of 18:2 acid into 20:4 acid and other 20 carbon fatty acids. The reverse of this hypothesis may explain the 18:2 and 20:4 content of yolk polar lipids in the prairie chicken. Apparently, this bird has a rather inefficient system of converting 18:2 into 20:4 acid. This view of a poor elongation desaturation system in prairie chickens could be strengthened by the significantly lower content of 22:6 acid in yolk phosphatides from this bird. This would imply that a similar situation exists to that which has been reported in the chick and hen (29): that the 18:3 acid is the precursor of docosahexaenoic acid. This could be the case in view of the absence of 22:6 acid in lipids from the experimental diets.

REFERENCES

1. Paleus, S., Bull. Soc. Chim. Biol., 49:917 (1967).
2. Erwin, J. and K. Bloch, Science 143:1006 (1964).
3. Bottino, N.R., R.E. Anderson and R.R. Reiser, Lipids 5:165 (1970).
4. Bottino, N.R., R.E. Anderson and R. Reiser, Fed. Proc. 27:362 (1968).
5. Breckenridge, W.C. and A. Kuksis, J. Lipid Res., 8:473 (1967).
6. Litchfield, C., Lipids 3:417 (1968).
7. Brockerhoff, H., R.J. Hoyle and N. Wolmark, Biochim. Biophys. Acta 116:67 (1966).
8. Storer, R.W., "Biology and Comparative Physiology of Birds, I," Academic Press, New York, New York, 1960, Chapter 3.
9. Edwards, H.M., Jr., J.C. Driggers, R. Dean and J.L. Carmon, Poultry Sci., 39:487 (1960).
10. Edwards, H.M., Jr., Ibid. 43:751 (1964).
11. Sell, J.L., S.H. Choo and P.A. Kondra, Ibid. 47:1296 (1968).
12. "Official Methods of Analysis of the Association of Official Agricultural Chemists," edited by W. Horwitz, Ninth edition, AOAC, Washington, D.C., 1960, p. 287.
13. Folch, J., M. Lees, G.H. Sloane Stanley, J. Biol. Chem., 226:497 (1957).
14. Murty, N.L., M.C. Williams and R. Reiser, J. Nutr. 72:451 (1960).
15. Privett, O.S., M.L. Blank and J.A. Schmit, J. Food Sci. 27:463 (1962).
16. Culp, T.W., R.D. Harlow, C. Litchfield and R. Reiser, JAOCS 42:974 (1965).
17. Blank, M.L., B. Verdino and O.S. Privett, Ibid. 42:87 (1965).
18. Luddy, F.E., R.A. Barford, S.F. Herb, P. Magidman and R.W. Riemenschneider, Ibid. 41:693 (1964).
19. Barford, R.A., F.E. Luddy and P. Magidman,

Lipids 1:287 (1966).
20. Saloma, A.E., F. Farr and J.R. Couch, Nutr. Rep. Int. 3:263 (1971).
21. Feldman, G.L., H.T. Johnson, T.W. Culp and R.H. Gowan, Poultry Sci., 41:1851 (1962).
22. Snedecor, G.W., "Statistical Methods," Fifth edition, Iowa State University Press, Ames, Iowa, 1965, pp. 237.
23. Duncan, D.B., Biometrics 11:1-42, (1955).
24. Reiser, R., J. Nutr. 40:429 (1950).
25. Fisher, H., and G.A. Leveille, Poultry Sci. 36:1116 (1957).
26. Ostrander, J.G., R. Jordan, W.J. Stadelman, J.D. Rogler and G.E. Vail, Ibid. 39:746 (1960).
27. Reiser, R., J. Nutr. 44:159 (1951).
28. Miller, E.C., H. Menge and C.A. Denton, J. Nutr. 80:431 (1963).
29. Murty, H.L., and R. Reiser, J. Nutr. 75:287 (1961).

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Egg Yolk Lipids and Maternal Diet in the Nutrition of Turkey Embryo

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ABSTRACT

Turkey hens were fed diets containing no added fat nor diets supplemented with soybean oil or neatsfoot oil. The composition of neutral and polar lipid fatty acids present in the unincubated turkey egg yolk was compared with that of those present in the yolk sac of the developing turkey embryo at different stages of development. Comparisons were made of the fatty acid fractions in the entire embryo homogenates, except liver and heart, which were analyzed separately. Changes in the relative amounts of the fatty acids are reported as affected by age of the embryo and by dietary lipids. The fatty acids from both the neutral and polar lipids which were utilized to the greatest extent for embryonic development were palmitoleic, oleic, linoleic, and linolenic, regardless of the dietary supplements. Arachidonic, tetracosenoic, and docosahexaenoic acids also were metabolized by the embryo. Saturated fatty acids, used by the embryo as development progressed, were palmitic, stearic, and arachidic acids. Analyses of the liver fatty acids showed that the C16:0, C16:1, C18:0, C18:1, and C20:4 acids in the neutral and polar lipids decreased with embryonic development and varied with the type of diet. The heart contained low levels of myristic, palmitic, stearic, arachidic, and arachidonic acids in the neutral lipids and palmitoleic and oleic acids in the polar lipids.

INTRODUCTION

The role of egg yolk lipids in the nutrition of the chick embryo has been studied by a number of investigators (1-10). Egg yolk fatty acids (FA) were found to be altered only slightly at 0, 8, 12, 16, and 20 days of incubation (2). The FA composition of 8 and 12 day embryos differed from the yolk FA, but after the 16th day of incubation, the differences between the chick embryo FA composition and the yolk FA decreased. A proportional increase in the utilization of unsaturated FA and a decrease in the utilization of saturated FA with the progress of embryonic development has been reported (3).

The 21-day chick embryo has been reported to utilize preferentially the polyunsaturated FA of the egg yolk, which increases the saturated FA content and decreases the polyunsaturated FA in the yolk remaining (4). Oleic acid also appeared to be utilized preferentially.

A periodic deposition of neutral lipids and phospholipids in the lateral thoracic fat organs of the chick has been reported to occur from the 13th to the 17th day of embryonic development (5). Liver phospholipids and neutral lipids were found to increase rapidly in the fat organs after the 17th day of development. There was little variation in the FA composition of embryo liver lipids from the 11th through the 19th day of development, except in the relative amounts of palmitate, stearate, and oleate (6).

The FA composition and concentration of individual phospholipids in livers of chick embryos have been studied at 13, 15, 17, and 21 days of embryonic development (7). The liver phospholipids contained greater proportions of phosphatidylethanolamine, phosphatidyl serine, and phosphatidyl glycerol than did yolk phospholipids, while the yolk phospholipids contained greater amounts of phosphatidyl choline. The yolk contained higher concentrations of palmitic and oleic acids in the phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl serine fractions than did the liver, while the liver contained higher concentrations of stearic, arachidonic, and docosahexaenoic acids in the fractions than did the yolk. The FA composition of glycerophosphatides in chick brains, liver, and yolk have been reported (8). Polyunsaturated FA constituted 30-40% of brain ethanolamine and serine glycerol phosphatides. Lecithin contained almost no polyunsaturated FA except for arachidonic acid. Brain, liver, and yolk ethanolamine and serine glycerol phosphatides were composed principally of the polyunsaturated FA C18:2, C20:4, and C22:6. During the third week of incubation, the C18:2 acid content doubled in all glycerolphosphatide fractions of the liver. A study was reported on the lipid metabolism of normal and vitamin B_{12}-deficient embryos (9). Between the 13th and 21st days of incubation, the liver triglycerides of normal embryos decreased in the concentration of C16:0 and

C22:6 acids while the concentration of C18:1 increased.

Tripalmitin metabolism has been studied with ^{14}C-label in both the glycerol and FA moieties (10). The triglyceride lost activity as it passed through the intestinal mucosa and ovary, while the phospholipids were transported from the ovaries to the eggs without a change in activity. During the incubation of the eggs, the triglycerides were transported from the yolk to the embryos without a change in activity, while the phospholipids were hydrolyzed during the transport from the yolk to the embryo which resulted in a loss of glycerol activity.

In an earlier report from this laboratory, it was shown that Beltsville Small White Turkey hens synthesize a significant amount of C18:1 when fed a fat free diet (FFD), as evidenced by the content of this FA in both yolk polar and neutral lipids (11). The C18:1 content of yolk lipids was not changed by adding a rich source of C18:1 to the hen diet. The C18:2 content of egg yolk polar and neutral lipids was increased significantly when the hen diet contained 30% soybean oil (SO). This increase occurred concurrently with a reduction in the C18:1 content in egg yolk polar and neutral lipids.

From the above review, it is apparent that the role of egg yolk fat in the nutrition of the chick embryo has been studied extensively. No references were found which described the metabolic changes in the turkey egg yolk lipids during the 28 day period of embryonic development. The purpose of the present investigation was to study the role of egg yolk lipids and maternal diet in the nutrition of the turkey embryo.

MATERIALS AND METHODS

Beltsville small white turkey hens were housed in individual cages and fed semisynthetic diets which contained no added fat or supplements of 3 or 30% SO or 3% neatsfoot oil (NO). A control group was fed a practical type milo-soybean meal diet (PD) which contained 2.26% total lipids. Neatsfoot oil was selected because of the high C18:1 content.

The composition of the diets used in this study and the analyses of the diets and of the unincubated eggs have been reported previously (11). The analyses of the diets are repeated in this publication (Table I). Yolk sacs were removed from the 10, 17, and 21 day old embryos and from hatched poults and analyzed, as previously described by Couch et al. (11). Three live embryos from each maternal treatment were removed from the shell at 10, 17, and 21 days of incubation. Hearts and livers were removed from three embryos from each dietary treatment at 17 and 21 days of incubation and from three hatched poults. Embryos, hearts, and livers were pooled within each group. The remaining portion of each of the three embryos were pooled and homogenized. The total lipids were extracted from the embryos, hearts, and livers by the method of Folch et al. (12). Fatty acid analyses on embryos, hearts, and livers were carried out, as previously outlined by Couch et al. (11).

TABLE I

Fatty Acid Composition of Diets

Fatty acid[a]	Experimental diets, %				
	FF[b]	3% SO[c]	30% SO[d]	3% NO[e]	PD[f]
16:0	24.6	12.3	12.7	18.1	17.4
16:1	4.3	0.7	0.4	5.0	3.9
18:0	9.0	6.4	5.3	6.9	3.0
18:1	28.0	27.9	25.4	50.8	27.0
18:2	29.6	43.9	47.4	12.6	29.9
18:3	0.0	4.9	5.6	2.6	2.0

[a]Per cent of total fat content.
[b]Diet containing no added fat.
[c]Diet containing 3% soybean oil.
[d]Diet containing 30% soybean oil.
[e]Diet containing 3% neatsfoot oil.
[f]Practical diet.

The data were analyzed by a split plot design of a factorial analysis of variance, according to Snedecor (13). Duncan's multiple range test (14) was used to compare each treatment mean with every other treatment mean. The test for significance in Tables II-VII was at the 5.0% probability level. Since the hearts were small and had to be pooled with only enough tissue for one FA analysis from each diet at each period, statistical significance between diets and periods was not determined on heart analyses.

RESULTS

Fatty Acid Content of Yolk and Yolk Sac Neutral and Polar Lipids

Stage of embryonic development–neutral lipids: The C18:0 content of the yolk sac neutral lipids did not change until the poult hatched (Table II). The highest value for this acid was found in the yolk sac from the hatched poult. The yolk sac from the hatched poult had a significantly higher content of C18:1 than the unincubated eggs or the yolk sac lipids before hatching. The C18:2 of the yolk sac neutral lipids decreased with embryonic development and was significantly lower in the yolk sac from the hatched poult than in the unincubated eggs. This indicated that the lino-

TABLE II

Average Fatty Acid Content of Neutral and Polar Lipids of Unincubated Egg and Yolk Sacs at 10, 17, and 21 Days of Incubation and from the Hatched Poult from all Diets[a]

Fatty acid	Unincubated egg, %	10 days, %	17 days, %	21 days, %	Hatched poult, %
Neutral lipids					
C16:0	26.5[2]	30.0[1,2]	30.7[1]	28.3[1,2]	28.6[1,2]
C18:0	5.2[2]	4.7[2]	5.4[2]	5.2[2]	8.5[1]
C16:1	8.9[1,2]	10.7[1]	9.7[1,2]	10.0[1,2]	7.7[2]
C18:1	41.3[2]	42.6[2]	42.5[2]	43.6[2]	46.3[1]
C18:2	13.6[1]	7.3[1,2]	6.5[1,2]	7.6[1,2]	3.6[1,2]
C18:3	1.2[1]	0.8[2]	0.7[2]	0.8[1,2]	0.4[2]
Polar lipids					
C16:0	25.0[2]	29.9[1]	29.4[1]	29.7[1]	25.3[2]
C18:0	21.5[1]	22.4[1]	21.0[1]	20.8[1]	23.1[1]
C16:1	3.1[1,2]	3.4[1,2]	3.9[1]	4.0[1]	2.7[2]
C18:1	29.6[1]	31.1[1]	31.2[1]	30.8[1]	30.3[1]
C18:2	11.6[1]	8.5[1]	7.6[1]	7.9[1]	10.2[1]
C18:3	1.0[1]	0.4[1]	0.7[1]	0.5[1]	0.3[1]
C20:4	3.4[1]	1.1[2]	1.5[2]	2.1[1,2]	3.2[1]

[a]Within each component, means bearing different superscripts are significantly different ($P<.05$).

leic acid content of the yolk was required by the developing embryo. There was a rather low content of C18:3, which also decreased with embryonic development.

Stage of embryonic development–polar lipids: The C16:0 content of the yolk sac polar lipids was significantly greater at 10, 17, and 21 days than that of the unincubated egg yolks and the yolk sac from the hatched poult (Table II). The C18:0 content of the yolk and yolk sacs essentially were unchanged during embryonic development. The C16:1 content of yolk sac polar lipids was decreased in the hatched poult, which indicates that this acid must have been utilized by the embryo during embryonic development from the level found in the unincubated egg. The C20:4 content of the yolk sac polar lipids decreased during the first 10 days of development but increased in the yolk sac from the hatched poult.

Effect of diet–neutral lipids: Yolk sac neutral lipids from hens fed FFD had the lowest level of C18:0, whereas those from hens fed 30% SO had the highest content of C18:0 (Table III). The C16:1 acid content of yolk sac neutral lipids was decreased significantly with diets containing 3 and 30% SO and in the yolk sacs from hens fed the PD. The C18:1 content of yolk sac lipids was significantly lower in the yolk sacs from hens fed the 3 and 30% SO diet,

TABLE III

Effect of Diet on the Average Fatty Acid Content of Neutral and Polar Lipids in Yolk Sacs[a]

Fatty acid	Fat free, %	3% Soy oil, %	30% Soy oil, %	3% Neatsfoot oil, %	Practical, %
Neutral lipids					
C16:0	27.1[1]	29.9[1]	29.4[1]	28.0[1]	29.8[1]
C18:0	4.4[3]	6.0[2]	9.7[1]	3.8[3]	5.0[2,3]
C16:1	12.8[1]	8.8[2]	4.7[3]	10.8[1,2]	9.9[2]
C18:1	49.0[1]	42.3[3]	29.7[4]	49.1[1]	46.2[2]
C18:2	3.3[2]	7.7[2]	17.4[1]	4.5[2]	5.7[2]
C18:3	0.6[2]	0.7[2]	1.5[1]	0.6[2]	0.4[2]
Polar lipids					
C16:0	27.5[1,2]	28.9[1]	23.8[2]	28.4[1]	30.8[1]
C18:0	18.7[3]	21.2[2]	29.7[1]	19.2[2,3]	20.0[2,3]
C16:1	5.0[1]	3.0[2]	1.6[3]	3.9[2]	3.5[2]
C18:1	37.0[1]	29.2[3]	20.2[4]	33.9[2]	32.8[2]
C18:2	4.2[3]	10.4[2]	16.8[1]	7.4[2,3]	7.1[2,3]
C18:3	1.1[1]	0.4[1]	0.3[1]	0.4[1]	0.4[1]
C20:4	2.3[1]	2.5[1]	2.6[1]	2.1[1]	1.8[1]

[a]Within each component, means bearing different superscripts are significantly different ($P<.05$).

and those from hens fed the 30% SO was significantly higher in C18:2 than those from any of the other treatments.

Effect of diet–polar lipids: The C16:0 content of yolk sac polar lipids from hens fed 30% SO was decreased significantly below that of all other diets, except those fed the FFD (Table III). The C18:0 content of yolk sac polar lipids from hens fed the 30% SO diet was significantly higher than those from the other treatments. The C16:1 acid content from yolk sac polar lipids was increased significantly in yolk sacs from hens fed the FFD and decreased in yolk sacs from those fed the 30% SO diet. The C18:1 acid content of yolk sac polar lipids was significantly higher when the hens had been fed the FFD and lowest in yolk sacs from those fed the 30% SO diet. The C18:2 content of yolk sac polar lipids was highest in yolk sacs from hens fed the 30% SO diet and lowest from those hens fed the FFD. No significant differences were noted between the levels of C18:3 and C20:4 acids in the polar lipids of the yolk sacs when the dietary treatments were considered.

Fatty Acid Content of Neutral and Polar Lipids of 10, 17, and 21 Day Embryos

Stage of embryonic development–neutral lipids: There was a decrease in the C18:0 content of embryo neutral lipids and an increase in the C18:1 and C18:2 acids at 17 and 21 days of development as contrasted with those of the 10 day embryo (Table IV). There was a significant decrease in the C20:4 acid at 21 days of development from the levels observed at 10 and 17 days.

Stage of embryonic development–polar lipids: The composition of C16:0, C18:0, and C18:3 content of polar lipids between 10 and

TABLE IV

Average Fatty Acid Content of Neutral and Polar Lipids of Total Embryos at 10, 17, and 21 Days of Embryonic Development (Hearts and Livers Removed from the 17- and 21-Day-Old Embryos)[a]

Fatty acid	10 days, %	17 days, %	21 days, %
Neutral lipids			
C14:0	1.2[1]	1.0[1,2]	0.9[2]
C16:0	28.6[2]	29.3[2]	32.2[1]
C18:0	15.4[1]	8.8[2]	7.3[2]
C20:0	1.0[1]	0.5[2]	0.2[2]
C26:0	2.5[1]	0.4[2]	0.2[2]
C16:1	5.8[1]	6.3[1]	6.6[1]
C18:1	27.9[2]	36.3[1]	38.9[1]
C18:2	4.1[2]	8.9[1]	7.8[1]
C18:3	0.6[1]	0.7[1]	0.8[1]
C20:4	1.7[1]	1.6[1]	0.5[2]
Polar lipids			
C14:0	1.2[1]	0.6[2]	0.5[2]
C16:0	38.0[1]	33.8[2]	35.4[1,2]
C18:0	21.9[1]	18.9[2]	21.0[1]
C16:1	3.9[1]	3.4[1]	3.2[1]
C18:1	23.7[1]	23.1[1]	20.6[2]
C24:1	0.8[2]	1.0[2]	1.4[1]
C18:2	3.3[2]	6.6[1]	6.2[1]
C18:3	0.6[1]	0.4[2]	0.5[1,2]
C20:4	2.9[2]	5.3[1]	4.8[1]

[a]Within each component, means bearing different superscripts are significantly different ($P<.05$).

TABLE V

Effect of Diet on the Fatty Acid Content of Neutral and Polar Lipids of Embryos from all Periods of Development[a]

Fatty acid	Fat free, %	3% Soy oil, %	30% Soy oil, %	3% Neatsfoot oil, %	Practical, %
Neutral lipids					
C14:0	0.9[2]	0.9[1,2]	1.2[1]	1.1[1,2]	1.0[1,2]
C16:0	28.9[2,3]	29.7[3]	26.3[3]	31.5[1,2]	34.0[1]
C18:0	9.0[2]	11.0[2]	13.5[1]	9.0[2]	10.0[2]
C16:1	8.0[1]	5.1[3]	4.7[3]	7.2[1,2]	6.1[2,3]
C18:1	41.3[1]	33.2[2]	24.5[3]	38.6[1,2]	34.3[2]
C24:1	1.2[1]	0.5[1]	0.6[1]	0.4[1]	0.2[1]
C18:2	2.6[3]	9.4[2]	14.9[1]	4.3[3]	3.3[3]
C18:3	0.6[1]	0.5[1]	0.6[1]	0.7[1]	1.1[1]
C20:4	1.2[1]	1.7[1]	1.5[1]	1.1[1]	0.7[1]
Polar lipids					
C16:0	36.5[1]	33.5[1]	36.6[1]	35.7[1]	36.3[1]
C18:0	18.4[3]	21.7[1,2]	23.6[1]	19.6[2,3]	19.6[2,3]
C16:1	3.9[1]	3.4[1,2]	2.7[2]	4.1[1]	3.4[1,2]
C18:1	26.2[1]	22.2[2]	18.3[3]	24.2[1]	21.4[2]
C18:2	4.5[2,3]	6.0[1,2]	7.5[1]	3.9[3]	5.0[2]
C18:3	0.6[1]	0.5[1,2]	0.5[2]	0.5[1,2]	0.5[1,2]
C20:4	3.1[2]	4.8[1,2]	3.0[2]	4.8[1,2]	5.7[1]
C22:6	0.3[1]	1.6[1]	0.9[1]	0.8[1]	1.3[1]

[a]Within each component, means bearing different superscripts are significantly different ($P<.05$).

TABLE VI

Average Fatty Acid Content of Neutral and Polar Lipids in Livers at 17 and 21 Days of Development and from the Hatched Poult from all Diets[a]

Fatty acid	17 days, %	21 days, %	Hatched poult, %
Neutral lipids			
C16:0	34.5[1]	18.4[2]	9.3[3]
C18:0	10.5[2]	16.4[1]	9.3[2]
C16:1	6.5[1]	5.2[2]	4.2[2]
C18:1	32.9[3]	48.1[2]	57.8[1]
C18:2	2.3[2]	1.8[2]	8.8[1]
C18:3	0.4[2]	0.4[2]	0.6[1]
C20:4	0.2[2]	0.0[2]	2.2[1]
C22:6	1.1[1]	1.0[1]	1.2[1]
Polar lipids			
C16:0	34.6[1]	28.3[2]	26.9[2]
C18:0	27.8[3]	41.3[1]	36.6[2]
C16:1	3.4[1]	1.5[2]	2.2[1,2]
C18:1	15.8[1]	15.1[1]	14.1[1]
C24:1	0.7[2]	1.1[1]	1.0[1,2]
C18:2	5.5[2]	4.7[2]	9.3[1]
C18:3	0.6[1]	0.4[1]	0.4[1]
C20:4	2.9[1]	1.5[2]	3.7[1]
C22:6	1.4[1]	0.8[2]	0.6[2]

[a]Within each component, means bearing different superscripts are significantly different (P<.05).

21 days of incubation varied (Table IV). The C18:2 and C20:4 content of the polar lipids increased significantly from 10-21 days of embryonic development. It is possible that the embryo was using both of these essential FA for metabolic processes during embryonic development. There was a significant decrease in the C18:1 acid at 21 days when compared with 10 and 17 day embryos.

Effect of diet–neutral lipids: Embryos from eggs produced by hens fed a 30% SO diet had a significantly higher level of C18:0 in the neutral lipids, a significantly lower level of C18:1, and a significantly higher level of C18:2 (Table V) than were found in embryos from any of the other diets. Embryos from eggs laid by hens fed 3% SO had a significantly higher level of C18:2 in the neutral lipids than did those from all other diets, except the hens fed the 30% SO diet. There was no difference in the C18:2 content of embryos from hens fed the FFD, NO, or PD. Embryos from eggs laid by hens fed the FFD had a significantly higher content of C18:1 in the neutral lipids than did embryos from hens fed the 3 or 30% SO diets and the PD but did not differ significantly from those from hens fed the 3% NO diet.

Effect of diet–polar lipids: Polar lipids of embryos from hens fed the 30% SO had a significantly lower content of C18:1 and a significantly higher content of C18:2 than did those from any of the other groups, with the exception of the C18:2 content of those from hens fed the 3% SO diet (Table V). Significantly higher levels of C18:1 were found in the polar lipids of embryos from hens fed the FFD and 3% NO, followed in order by the 3% SO and PD. The C18:2 content of the polar lipids of embryos from hens fed the 3% NO was significantly lower than that of those from any

TABLE VII

Effect of Diet on the Average Fatty Acid Content of Neutral and Polar Lipids in Livers from all Periods of Development[a]

Fatty acid	Fat free, %	3% Soy oil, %	30% Soy oil, %	3% Neatsfoot oil, %	Practical, %
Neutral lipids					
C14:0	0.9[1]	0.8[1]	1.0[1]	1.2[1]	1.0[1]
C16:0	16.1[2]	23.6[1]	20.7[1,2]	21.4[1,2]	21.8[1,2]
C18:0	8.6[2]	13.4[1]	14.7[1]	11.6[1]	12.2[1]
C16:1	5.5[1,2]	4.7[2]	4.5[2]	6.7[1]	5.1[2]
C18:1	55.6[1]	46.0[2]	33.3[3]	48.4[2]	48.0[2]
C24:1	0.4[1]	0.3[1]	1.0[1]	1.1[1]	0.4[1]
C18:2	2.8[2,3]	3.6[2]	11.8[1]	1.8[3]	1.6[3]
C18:3	0.1[2]	0.6[1]	0.7[1]	0.5[1]	0.4[1,2]
C20:4	0.4[2]	0.5[2]	2.9[1]	0.3[2]	0.2[2]
C22:6	1.7[2]	0.4[2]	2.5[1]	0.2[2]	0.0[2]
Polar lipids					
C16:0	29.8[2]	32.8[1]	27.1[3]	27.5[3]	32.5[1]
C18:0	32.5[2]	36.0[1,2]	33.7[2]	33.6[2]	40.3[1]
C16:1	2.1[2]	1.8[2]	1.8[2]	4.6[1]	1.6[2]
C18:1	19.4[1]	13.4[2,3]	10.5[3]	17.7[1]	14.0[2]
C24:1	0.9[2]	1.1[1,2]	0.4[3]	1.5[1]	0.7[2,3]
C18:2	4.9[2]	5.6[2]	13.7[1]	5.4[2]	3.0[3]
C18:3	0.6[1]	0.4[1]	0.3[1]	0.5[1]	0.6[1]
C20:4	3.3[2]	2.1[2]	5.4[1]	2.3[2]	0.6[3]
C22:6	0.8[2]	0.7[2]	2.1[1]	0.5[2]	0.6[2]

[a]Within each component, means bearing different superscripts are significantly different (P<.05).

other group, with the exception of those from hens fed FFD. There was no significant difference in the C18:2 content of embryos from hens fed the FFD, 3% SO, and PD. The polar lipids of embryos from hens fed FFD and 30% SO contained significantly lower levels of C20:4. No consistent pattern was apparent in the values for the other FA, except that the C16:0 and C22:6 content of polar lipids did not differ significantly, regardless of the dietary source.

Fatty Acid Content of Neutral and Polar Lipids of Livers at 17 and 21 Days of Development and from the Hatched Poult

Stage of development–neutral lipids: The C16:0 content of liver neutral lipids decreased significantly from 17-21 days of development and in the hatched poult 7 days later (Table VI). There is no explanation for the fact that the C18:0 content of the liver neutral lipids increased from 17-21 days and decreased again in the hatched poult. The C16:1 content of the liver neutral lipids decreased significantly at 21 days and in the liver from the hatched poult, although numerically the decrease was not great. There was a significant increase in the C18:1 content of the liver neutral lipids from 17-21 days and again from 21 days-hatching. The C18:2 content of the liver neutral lipids increased ca. four-fold from the 17th and 21st days to the 28th day of development.

Stage of development–polar lipids: The C16:0 content of the liver polar lipids was significantly lower at 21 days of development and in the liver from the hatched poult (Table VI). The C18:0 content of embryo liver polar lipids was significantly greater at 21 days of development and in the liver from the hatched poult. The C18:2 content of the liver polar lipids was significantly greater in the hatched poult than was that of embryos at 17 and 21 days of development. The C20:4 content of embryonic polar lipids was significantly less at 21 days than at 17 days of development and in the liver from the hatched poult.

Effect of diet–neutral lipids: Embryos from hens fed the FFD had a significantly lower level of C18:0 in the liver neutral lipids with no difference in those from the other four diets (Table VII). Embryos from hens fed the FFD had a significantly higher level of the C18:1 acid in the liver neutral lipids. There was no difference in the C18:1 content in the liver neutral lipids of embryos from hens fed the 3% SO, 3% NO, and PD. The lowest level of C18:1 was found in liver neutral lipids of embryos from hens fed the 30% SO diet and the highest level in liver neutral lipids of embryos from hens fed the PD. The liver neutral lipids of embryos from hens fed the 30% SO diet had a significantly higher level of C18:2 and C20:4 than did the liver neutral lipids of embryos from hens fed any of the other diets.

TABLE VIII

Average Fatty Acid Content of Neutral and Polar Lipids from Hearts at 17 and 21 Days of Development and from the Hatched Poult

Fatty acid	17 days, %	21 days, %	Hatched poult, %
Neutral lipids			
C14:0	1.4	1.8	1.2
C16:0	31.2	29.8	28.0
C18:0	14.2	17.4	12.1
C20:0	1.3	3.2	0.9
C26:0	1.0	0.9	0.9
C16:1	5.6	5.2	6.7
C18:1	29.4	25.1	37.7
C18:2	3.1	3.8	5.0
C20:4	1.4	1.5	0.4
C22:6	0.5	0.3	1.8
Polar lipids			
C14:0	0.5	0.4	1.2
C16:0	34.0	38.8	34.0
C18:0	26.1	25.7	29.6
C16:1	2.3	1.7	1.7
C18:1	22.0	19.7	19.9
C24:1	1.0	0.9	1.1
C18:2	4.0	3.8	3.2
C20:4	1.9	1.5	0.9

Effect of diet–polar lipids: Embryos from hens fed 30% SO and 3% NO had a significantly lower level of C16:0 in the polar lipids than did the livers of embryos from hens fed the FFD, 3% SO, or PD (Table VII). Embryos from hens fed 3% NO had a significantly higher level of C16:1 in the polar lipids than did those from hens fed any of the other diets. The embryo polar lipids from hens fed the 30% SO diet had a significantly higher level of C18:2, C20:4, and C22:6 than did those from hens fed any of the other diets. Embryonic polar lipids from hens fed the FFD and the 3% NO diet had the highest level of C18:1. This acid is readily synthesized by the hen and deposited in the yolk in diets low in polyunsaturated FA (4). The C18:2, C20:4, and C22:6 content of embryonic polar lipids from hens fed the 30% SO diet was significantly higher than that of embryos from any of the other groups.

Fatty Acid Content of Heart Neutral Lipids at 17 and 21 Days of Development and from the Hatched Poult

Stage of development–neutral lipids: The C18:0 content of the heart neutral lipids increased at 21 days of development and decreased in the hatched poult (Table VIII). The C20:0 content of the heart neutral lipids increased slightly at 21 days and decreased in

TABLE IX

Effect of Diet on the Average Fatty Acid Content of Neutral and Polar Lipids of Hearts from all Periods of Development

Fatty acid	Fat free, %	3% Soy oil, %	30% Soy oil, %	3% Neatsfoot oil, %	Practical, %
Neutral lipids					
C14:0	1.9	1.3	1.7	1.2	1.3
C16:0	25.9	29.9	30.2	30.5	31.9
C18:0	12.2	16.2	17.7	12.7	14.0
C20:0	3.0	1.7	1.3	1.4	1.6
C26:0	0.9	0.3	0.9	1.3	1.4
C16:1	5.8	4.5	6.0	7.7	5.2
C18:1	34.8	31.9	22.5	32.5	31.9
C18:2	3.4	4.2	5.5	2.8	4.0
C18:3	1.1	0.8	0.7	0.9	0.6
C20:4	2.1	0.7	1.1	0.8	0.7
C22:6	0.5	0.0	0.8	0.5	2.0
Polar lipids					
C14:0	0.7	1.8	0.2	0.4	0.2
C16:0	34.3	35.0	38.0	34.4	36.2
C18:0	25.9	29.4	28.6	25.0	26.6
C16:1	2.5	1.2	1.8	2.3	1.6
C18:1	24.8	17.7	15.2	24.4	20.5
C24:1	0.6	1.9	0.8	0.9	0.8
C18:2	2.6	2.5	6.0	3.0	4.1
C20:3	0.2	1.3	0.5	0.5	0.4
C20:4	1.3	0.9	2.0	1.3	1.5

the hearts from the hatched poults. There was an increase in the C18:1 and in the C18:2 of the heart neutral lipids in the hatched poult, as contrasted with those of the embryos at 17 and 21 days of development.

Stage of development–polar lipids: There was little difference in the FA content of the heart polar lipids at 17 and 21 days of development and in the hatched poult (Table VIII). There was an increase in the C16:0 content of the heart polar lipids at 21 days of development, which was followed by a decrease in the heart polar lipids of the hatched poult. There was an increase in the C18:0 content of heart polar lipids in the hatched poult.

Effect of diet–neutral lipids: The diet exerted little effect on the FA content of the heart neutral lipids (Table IX). There was an increase in the C18:0 content of the heart neutral lipids of embryos from eggs laid by hens fed 3 and 30% SO and an increase in the C16:0 content of the heart polar lipids of embryos from hens fed 3% SO, 30% SO, 3% NO, and PD. There was a decrease in the C18:1 content of heart neutral lipids of embryos from hens fed the 30% SO diet and an increase in the C18:2 content of embryo heart neutral lipids.

Effect of diet–polar lipids: The diet of the turkey hen exerted a relatively small effect on the FA content of the embryo heart polar lipids (Table IX). There was a decrease in the C18:1 content of heart polar lipids of embryos from eggs laid by hens fed 3 and 30% SO. There was an increase in the C18:2 content of the embryo heart polar lipids from hens fed the 30% SO diet, which may be traceable to the high C18:2 content of this diet.

DISCUSSION

Significant changes in the FA content of yolk sac, embryo, and liver FA occurred in the mono- and polyunsaturated FA in both the neutral and polar lipids. Yolk sac content of these FA tended to decrease, whereas the embryo and liver content of these FA increased as the embryo developed to maturity. This agrees with data reported on studies with the chick embryo (4) in which it was stated that the chick embryo preferentially utilized the polyunsaturated triglycerides of the egg yolk during development.

The effect of diet on the FA content of the egg yolk neutral and polar lipids from these hens has been reported previously (11). It can be stated further that the diet of the turkey hen had a significant effect on the deposition of dietary FA in the yolk sac, embryos, and livers. Less effect was noted from the effect of diet on the FA in the heart neutral and polar lipids.

The turkey embryo preferentially uses palmitoleic, oleic, linoleic, and arachidonic acids for development. The most significant of these is the usage of linoleic and arachidonic acids. The diet of the turkey hen had a significant effect on the dietary deposition of the FA in

the total embryos and embryonic liver neutral and polar lipids.

REFERENCES

1. Eaves, E.C., J. Physiol. 40:451 (1964).
2. Donaldson, W.E., Poultry Sci. 43:784 (1964).
3. Nakamura, T., T. Takanhashi and S. Kawai, Gifu Daigaku Nogakubu Kenkyu Hokoku 26:170 (1968).
4. Isaacks, R.E., R.E. Davies, T.M. Ferguson, R. Reiser and J.R. Couch, Poultry Sci. 43:113 (1964).
5. Feldman, G.L., L.M. Churchwell, T.W. Culp, F.A. Doyle and H.T. Jonsson, Ibid. 41:1232 (1962).
6. Feldman, G.L., and C.K. Grantham, Ibid. 43:150 (1964).
7. Noble, R.C., and J.H. Moore, Can. J. Biochem. 45:627 (1967).
8. Miyamoto, K., L.M. Stephanides and J. Bernshon, J. Lipid Res. 7:664 (1966).
9. Noble, R.C., and J.H. Moore, Biochem. J. 95:144 (1965).
10. Budowski, R., N.R. Bottino and R. Reiser, Arch. Biochem. Biophys. 93:483 (1961).
11. Couch, J.R., B.M. Cornett, T.M. Ferguson and C.R. Creger, Nutr. Repts. Int. 1:83 (1970).
12. Folch, J., M. Lees and G.H. Sloane-Stanley, J. Biol. Chem. 226:497 (1957).
13. Snedecor, G.W., "Statistical Methods," Iowa State College Press, Ames, 1956.
14. Duncan, D.B., Biometrics 11:1 (1955).

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Lipids of Cultured Hepatoma Cells: I. Effect of Serum Lipid Levels on Cell and Media Lipids[1]

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ABSTRACT

Minimal deviation hepatoma cells were cultured as monolayers to confluency in roller flasks containing modified Swim's medium, supplemented with decreasing amounts of serum, lipid-free serum, and lipid-free serum containing added fatty acids. Good cell growth was observed until serum levels fell below 5% of the medium. Media containing lipid-free serum or lipid-free serum plus linoleic or palmitic acids did not support good growth. Lipids were extracted from cells; media, obtained during the first and last half of the incubation period, resolved into neutral and phospholipid fractions; fatty acid composition of each fraction analyzed by gas liquid chromatography; and lipid class distributions compared by thin layer chromatography. The data showed that the media contained more neutral lipids and phospholipids after incubation than initially, indicating that minimal deviation hepatoma cells excreted lipids into the media. The class composition of the excreted lipids resembled that of the serum. A comparison of media, cells, and serum fatty acid compositions indicated that the lipids secreted into the media were of cellular origin. Although some differences were noted, in general, cells grown on the nine different media had the same ca. neutral lipid and phospholipid class and fatty acid compositions. In contrast, dramatic differences were observed in the class and fatty acid compositions of the serums from that of the cells and media. These results indicate that exogenous serum lipids had little influence on cellular class and fatty acid compositions of the minimal deviation hepatoma cells. This neoplasm did not contain detectable levels of glyceryl ether diesters, indicating that this compound is not characteristic of all tumors. Lipid class profiles and fatty acid compositions of cells grown on various media suggest that the minimal deviation hepatoma cells can synthesize most, if not all, neutral lipid and phosphoglyceride classes found in liver.

INTRODUCTION

The last decade of biochemical research has brought lipids from a point at which they were considered a good source of energy to the present recognition of their importance in many biological processes. Lipids are major components of all membranes (1-3), essential components of many enzyme systems (4), and precursors of a potent class of hormones (5,6). Lipids are involved in biological mineralization (7), ion transport (8), sugar transport (9), and numerous other important cellular functions. Some investigators in cancer research have recognized the possible direct or indirect involvement of lipids in neoplasia and have obtained data to indicate numerous differences between the lipid metabolism of normal and neoplastic tissue. Much data on the biochemistry and metabolism of tumor lipids, compiled from many laboratories, has appeared recently (10).

Although differences in lipid metabolism occur between normal and neoplastic tissue, few consistently appear in all tumors because of their differences in origin, stage of neoplasia, nutritional state of host, and numerous other factors. The development by Morris and colleagues (11) of a series of "minimal" deviated hepatomas eliminated many of the difficulties in deciding which normal tissue should be compared with neoplasm data. The host effect on metabolism of a minimal deviation hepatoma also can be eliminated by growing hepatoma cells in tissue culture.

Structural and metabolism studies on lipids, derived from minimal deviation hepatoma cells grown in culture, have been limited. The incorporation of activity from various radioactive substrates into the nonsaponifable lipids (12) and the composition and biosynthesis of gangliosides in cultured minimal deviation hepatoma cells have been reported (13). Recently Ontko (14) and Goodridge (15) described fatty acid biosynthesis and metabolism studies obtained with normal liver hepatocytes cultured for relatively short periods of time. The lack of lipid data from minimal deviation hepatoma cells, grown in culture which have numerous advantages, and the potential availability of

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TABLE I

Quantity of Neutral Lipids and Phospholipids Derived from Minimal Deviation Hepatoma Cells Grown on Media Containing Varying Amounts of Serum and Lipid[a]

Basal medium supplement[b]	Cell numbers x 10^6[c]	Doubling time (days)	μg of lipid/ 10^6 cells		μg of lipid/ mg of lipid-free dry wt		Percentage	
			PL[d]	NL[d]	PL	NL	PL	NL
20% Bovine serum + 5% fetal calf serum	446	1.18	37	21	106	62	63	37
10% Bovine serum + 5% fetal calf serum	665	1.18	23	15	—	—	57	43
5% Bovine serum + 5% fetal calf serum	635	1.32	31	24	110	87	60	40
5% Fetal calf serum	565	1.44	17	12	119	80	60	40
5% Bovine serum	384	1.53	18	8	87	39	69	31
2.5% Fetal calf serum	342	2.07	22	7	—	—	77	23
5% Lipid-free fetal calf serum	194	2.5	18	7	71	30	71	29
5% Lipid-free fetal calf serum + palmitic acid[d]	94	5.5	45	47	208	222	49	51
5% Lipid-free fetal calf serum + linoleic acid[e]	92	3.5	53	55	107	111	49	51

[a]All data obtained from cells grown in roller cultures for five-six days.

[b]Basal medium was a modified Swim's 77.

[c]Total number of cells harvested from two roller culture flasks and analyzed as a single sample.

[d]PL = phospholipids, NL = neutral lipids.

[e]37.5 μg per ml of medium.

comparable data from normal hepatocytes prompted us to initiate a study to examine the metabolism and structure of lipids at the molecular species level from the minimal deviation hepatoma 7288C(HTC) grown in tissue culture (16).

This article represents the first in a series on the data obtained with cultured HTC cells and a solid minimal deviation hepatoma 7288CTC obtained by the reintroduction of HTC cells into a host (17). These data show the effects of exogenous serum lipid levels on growth rate of HTC cells and class and fatty acid compositions of cellular and media neutral lipid and phospholipids.

MATERIALS AND METHODS

Cells

Starter cultures of HTC cells were obtained from W.D. Noteboom, Department of Biochemistry, University of Missouri, who had obtained the cells from G.M. Tomkins, the developer of this line of cultured minimal deviation hepatoma cells (16,18). Cells were obtained at the 17th passage. Stock cultures were maintained on a modified Swim's 77 medium supplemented with 20% bovine serum and 5% fetal calf serum. The medium modification included the addition of glutamine (292 mg/l), sodium bicarbonate (500 mg/l), calcium chloride dihydrate (265 mg/l), Sodium Penicillin G (60 mg/l), L-cystine (12 mg/l), and phenol red (5 mg/l). All media were sterilized by filtration through 0.2 μ pore size filters.

Gram quantities of cells grown on various amounts of serum and lipids (Table I and II) were obtained from roller cultures. Cells (30-50 million) were planted in two 10 x 20 cm glass roller culture bottles containing ca. 100 ml of medium each and incubated at 37 C. The roller bottles were rotated at the rate of 0.8 RPM for the first four-six hr to ensure an even distribution of cells, after which time the rate was reduced to 0.25 RPM. The media were changed on the second or third day of incubation. After five-six days, when the cells had reached confluency or failed to grow further, the medium from the second half of incubation was collected. The cells were released enzymatically from the roller flasks, counted, washed with serum-free Swim's 77 medium, and transferred to tared lyophilization flasks. Medium from the first half or last half of incubation was centrifuged for 6 x 10^6 g-min to sediment any particulate matter.

TABLE II

Accumulation of Phospholipids and Neutral Lipids in the Media of Cultured Minimal Deviation Hepatoma Cells

Basal medium supplement	Cells[a]			Media (mg)					
						Lipid gain during incubation			
	Numbers x 10^6	Lipid (mg)		Initial lipid content/200 ml		First half[b]		Last half[b]	
		PL	NL	PL	NL	PL	NL	PL	NL
20% Bovine serum + 5% fetal calf serum	446	16.5	9.6	47.7	56.5	—	—	+24.8[c]	+17.8[c]
10% Bovine serum + 5% fetal calf serum	665	15.0	10.3	21.6	26.7	+2.9	+16.2	+19.9	+19.8
5% Bovine serum + 5% fetal calf serum	635	19.4	15.3	15.1	16.6	—	—	+15.9	+8.2
5% Fetal calf serum	565	9.8	6.6	4.3	3.3	—	—	+3.2	+0.8
5% Bovine serum	384	7.0	3.2	10.8	13.3	+10.6	+8.9	+17.7	+11.5
2.5% Fetal calf serum	342	7.5	2.3	2.2	1.7	+3.2	−0.4	+6.6	+0.9
5% Lipid-free fetal calf serum	194	3.5	1.5	1.2	0	+2.8	+0.5	+2.2	+1.4
5% Lipid-free fetal calf serum + palmitic acid[d]	94	4.2	4.4	1.2	7.5[d]	+3.7	+0.6	+6.6	+1.1
5% Lipid-free fetal calf serum + linoleic acid[d]	92	4.9	5.1	1.2	7.5[d]	+4.4	+2.0	+3.7	+3.4

[a]Cell numbers represent the total number of cells harvested from two roller flasks after five-six days of incubation and analyzed as a single sample. Each flask contained between 15 and 25 million cells at the start of incubation.

[b]Neutral lipid (NL) and phospholipid (PL) weights represent the difference between lipids known to be in the media at the start of incubation (given in columns 4 and 5) and that found in the first or last half incubation media.

[c]Represents the total media lipids for both first and last half (media not changed).

[d]37.5 μg per ml of medium.

Lipid Extraction and Fractionation

Lipid-free fetal calf serum was prepared by sonicating lyophilized serum with 20-30 volumes of chloroform-methanol, 2:1 (v/v), until a homogenous suspension, free of clumps, was obtained. Solvent was removed by filtration through a fine porosity fitted disc glass funnel and washed with another 50 volumes of chloroform-methanol. The chloroform-methanol extracts were combined, evaporated, and weighted to determine extent of lipid extraction. The lipid-free protein was transferred to a 100 ml pear-shaped flask with chloroform and methanol and the solvent flash evaporated, leaving a thin film on the wall of the flask which facilitated the removal of the last traces of solvent under high vacuum. When linoleic and palmitic acid were added to the medium, they were introduced at this point before the solvent was flash evaporated. The lipid-free fetal calf serum or lipid-free serums containing added fatty acids were reconstituted in either water or Swim's 77 medium.

Lyophilized cells, media, and serums for lipid determinations were extracted by the Bligh and Dyer procedure (19) to obtain total lipids. A second extraction was performed by readjusting chloroform-methanol concentration to give a single phase again. Each time the suspension was sonically disrupted for ca. five min to facilitate lipid extraction. Neutral lipids were separated from the phospholipids by silicic acid chromatography (20). Percentages of neutral lipid and phospholipid fractions were determined gravimetrically. A qualitative examination of the neutral lipid fraction was made by thin layer chromatography (TLC) on adsorbent layers of Silica Gel G, developed in a solvent system of hexanediethyl ether-acetic acid 80:20:1, v/v. The qualitative and quantitative examination of individual phospholipid classes was carried out by TLC on adsorbent layers of Silica Gel HR, developed in a solvent system of chloroform-methanol-acetic acid-0.9% saline, 50:25:8:4, v/v.

Other Procedures

Phosphorous was determined by the method of Rouser et al. (21). Chromatoplates used for qualitative analyses were sprayed with sulfuric acid, charred, and documented by photography. Class identification was based upon the

use of phosphorous and ninhydrin spray reagents (22), gas liquid chromatography (GLC) of hydrolysis products, and cochromatography with authentic phospholipid and neutral lipid standards in two or more TLC solvent systems. Methyl esters of the total neutral lipid and phospholipid fractions were prepared by acid catalyzed esterification and analyzed by GLC, as described previously (23).

Materials

Single lots of fetal calf serum, bovine serum, and Swim's 77 medium obtained from Grand Island Biological Co., Grand Island, N.Y., were used for these experiments. Fatty acids and neutral lipid standards were purchased from Nu-Check-Prep, Inc., Elysian, Minn. Phospholipid standards were purchased from Supelco, Inc., Bellefonte, Pa. All solvents were glass-distilled and obtained from Burdick and Jackson Laboratories, Muskegon, Mich. Other chemicals and reagents used were reagent grade, or better, and used without further purification.

RESULTS

Cell Total Lipids

Numbers of cells harvested, doubling time, and quantity of neutral lipids and phospholipids, derived from HTC cells cultured on Swim's 77 medium containing varying amounts of serum and lipids, are given in Table I. The doubling time, which included the lag phase, increased when serum levels decreased below 10%, and growth was poor in media containing less than 5% serum. Removal of lipids from the serum further increased the doubling time, and the addition of either palmitic or linoleic acid to the lipid-free fetal calf serum did not improve growth. Except for the growth conditions where fatty acids were added, the phospholipids represented between 60 and 70% of the total cell lipids. The quantity of phospholipid/given number of cells or/given amount of dry wt showed less variation than the neutral lipids.

Media Total Lipids

The total lipid of fetal calf and bovine serums represented 2.2 and 4.4%, respectively, of the dry wt. Fetal calf serum and bovine serum contained 3.6 and 5.6% dry wt, respectively, of the wet wt. These values were used to calculate the amount of neutral lipid and phospholipid in the original medium of each experiment. When chloroform-methanol was used to extract the lipids from fetal calf serum for the experiments using lipid-free fetal calf serum, only 85% of the lipids were removed relative to the amount obtained by the Bligh and Dyer extraction procedure. The difference was probably due to nonlipid components being extracted by the Bligh and Dyer procedure. However, to remove bias from the data, the chloroform-methanol extracted serum was assumed to contain 15% unextracted phospholipid, and this was taken into account in the calculation of accumulated lipid. The differences between the amount of lipid in the media at the start of the incubations and that found in the media after the first or last half of the incubations are given in Table II. The media from all HTC cell growth conditions showed a gain in the neutral lipid (one exception) and phospholipid fractions. Generally, the last half of the incubation showed more lipid accumulation than the first half. Table II shows that the quantity of lipid found in the media exceeds that found in the HTC cells in all cases where media were collected from both incubation periods.

TLC of Cell Neutral Lipids

The distribution of major neutral lipid classes derived from HTC cells cultured in Swim's 77 medium, supplemented with various levels of serum and lipids, is shown in Figure 1, along with the distribution of neutral lipids found in bovine and fetal calf serums. Sterol esters, triglycerides, free fatty acids, and sterols were the major neutral lipid classes present in HTC cells from all experiments. Triglycerides appeared to be the most abundant component in all growth conditions, except one, while the serum triglyceride levels in both serums were low. Cells grown on lipid-free fetal calf serum contained all the major neutral lipid classes. Supplementation of the lipid-free fetal calf serum with free palmitic or linoleic acids did not elevate the free fatty acids in the cells above other growth conditions but did appear to reduce the level of sterol esters. Glyceryl ether diesters were not detected on these heavily loaded plates nor when even heavier loads were examined.

TLC of Cell Phospholipids

Figure 2 shows the distribution of the major phospholipid classes derived from HTC cells grown on media containing various levels of serum and lipids. The phospholipid class distribution of bovine and fetal calf serums shown for comparison was quite different from the class distribution of cells obtained during the various growth conditions. Phosphatidylethanolamine, absent from the serums, and phosphatidylcholine, the major phospholipid class of the serums, were the two major cellular

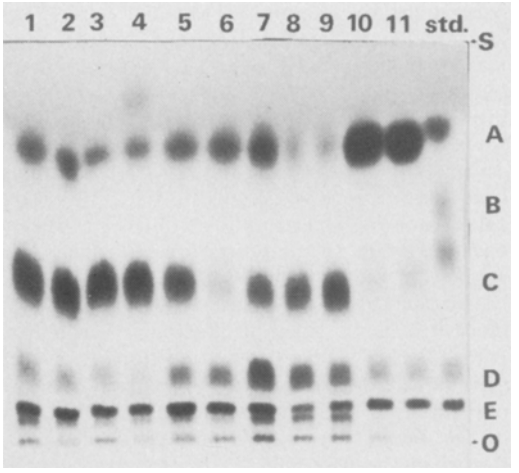

FIG. 1. Thin layer chromatoplate of neutral lipids derived from minimal deviation hepatoma cells cultured on a modified Swim's 77 medium supplemented with: lane 1, 20% bovine serum plus 5% fetal calf serum; lane 2, 10% bovine serum plus 5% fetal calf serum; lane 3, 5% bovine serum plus 5% fetal calf serum; lane 4, 5% fetal calf serum; lane 5, 2.5% fetal calf serum; lane 6, 5% bovine serum; lane 7, 5% lipid-free fetal calf serum; lane 8, 5% lipid-free fetal calf serum plus palmitic acid; and lane 9, 5% lipid-free fetal calf serum plus linoleic acid. Lanes 10 and 11 represent neutral lipids from fetal calf serum and bovine serum, respectively. A neutral lipid standard is represented by standard. Neutral lipid classes are: A, sterol esters; B, glyceryl ether diesters; C, triglycerides; D, free fatty acids; and E, sterols. The origin and the solvent front are marked by O and S, respectively. Each lane, except the standard, was spotted with 200 μg of sample and developed in a solvent system of hexane-diethyl ether-acetic acid 80:20:1 (v/v).

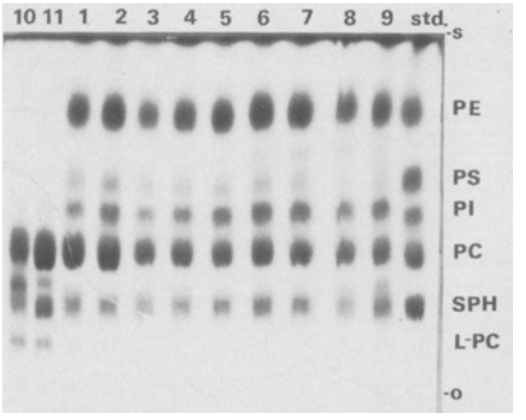

FIG. 2. Thin layer chromatoplate of phospholipids isolated from minimal deviation hepatoma cells cultured on a modified Swim's 77 medium supplemented with various amounts of serums and lipids. The numbered lanes correspond to culture conditions described in Figure 1. Phospholipid classes are: PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; SPH, sphingomyelin; and L-PC, lyso-phosphatidylcholine. The origin and solvent front are indicated by O and S, respectively. Each lane, except the standard, was spotted with 300 μg of sample and developed in a solvent system of chloroform-methanol-acetic acid – 0.9% saline, 50:25:8:4 (v/v) when the relative humidity was below 35%.

phospholipids. HTC cells did not contain more than trace levels of lyso-phosphatidylcholine, while both serums contained relatively high levels. Generally, gross differences in the phospholipid class distribution could not be detected by visual comparison on TLC, even for the phospholipids derived from cells grown in media containing lipid-free fetal calf serum.

TLC of Media Neutral Lipids

The distribution of neutral lipid classes found in the media was different from cellular neutral lipids but similar to the distribution of the neutral lipid classes from the serums. Sterol esters were the major lipid class under all growth conditions, except when lipid-free fetal calf serum was supplemented with linoleic acid. Triglycerides, a major cellular neutral lipid, were present at low levels in the media. Media supplemented with lipid-free fetal calf serum contained the same lipid classes as the media from cells grown in the presence of serum. Like the cells, the media did not contain detectable levels of glyceryl ether diester. Distribution of neutral lipid classes from the first half incubation media resembled the last half distribution.

TLC of Media Phospholipids

The distribution of media phospholipids was strikingly different from the cellular pattern but similar to serum phospholipid patterns. Phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol were present in only trace amounts or absent, while phosphatidylcholine and sphingomyelin were the major phosphatides present. An unidentified compound migrating between phosphatidylethanolamine and phosphatidylserine did not contain phosphorous. This compound appeared to increase in concentration as serum and lipid levels decreased. The distribution of media phospholipids from first and last incubations were similar, as judged from TLC.

Fatty Acid Composition of Cellular Neutral Lipids

The percentage distribution of fatty acids obtained from the total neutral lipids of fetal calf serum, bovine serum, and HTC cells grown on various serum and lipid levels is given in Table III. The fatty acids derived from fetal calf and bovine serum neutral lipid were strikingly different. Fetal calf serum neutral lipids were characterized by high levels of 16:0, 18:1,

TABLE III

Fatty Acid Composition of Total Neutral Lipids Derived from Serums and Minimal Deviation Hepatoma Cells Grown on Media Containing Various Amounts of Lipid

Serums and basal medium supplement	Fatty acid percentages[a,b]									
	14:0	16:0	16:1	18:0	18:1	18:2	20:1	20:2	20:4	>20:4
Serums										
Fetal calf serum	T[c]	23.5	7.1	4.5	33.8	9.3	0.9	—	12.5	6.4
Bovine serum	T	9.8	3.3	5.4	12.8	57.4	4.2	—	4.1	T
Minimal deviation hepatoma										
20% Bovine serum + 5% fetal calf serum	0.5	7.4	5.8	5.7	66.4	8.1	3.7	—	1.4	1.0
10% Bovine serum + 5% fetal calf serum	1.2	10.2	7.1	5.9	61.7	7.2	5.6	—	1.1	T
5% Bovine serum + 5% fetal calf serum	1.4	11.6	8.8	5.3	63.3	4.1	4.4	—	1.2	T
5% Fetal calf serum	0.8	10.6	6.9	5.8	67.9	1.6	5.0	—	1.3	T
5% Bovine serum	T	9.1	4.5	7.4	63.0	10.2	3.7	—	3.9	T
2.5% Fetal calf serum	T	8.0	8.0	5.3	65.6	4.2	5.3	—	2.2	T
5% Lipid-free fetal calf serum	1.6	8.7	8.3	5.0	65.7	2.5	5.1	—	0.7	T
5% Lipid-free fetal calf serum + palmitic acid[d]	0.5	8.4	6.0	8.7	64.3	4.6	3.9	—	2.0	T
5% Lipid-free fetal calf serum + linoleic acid[d]	T	6.7	2.1	5.8	16.7	49.7	T	8.1	6.5	2.4

[a]Difference between the sum of the fatty acid percentages in each row and 100% represents the sum of minor components not given in the table.

[b]Percentages represent the mean of duplicate analyses on a single pooled sample.

[c]T = less than one half per cent.

[d]37.5 μg per ml of medium.

20:4, and those acids with retention times greater than 20:4, whereas bovine serum was characterized by a high level (ca. 60%) of 18:2 acid. Cellular neutral lipid composition differed from either of the serums. With one exception, cellular neutral lipid fatty acid percentages were not changed significantly by reduced amounts of serum and lipid in the medium. Palmitic and stearic acids remained relatively constant under all growth conditions, whereas 18:1 acid increased as serum and lipid levels were decreased. Cells, grown on medium supplemented with lipid-free fetal calf serum, contained the lowest level of 18:2 and 20:4 acids. The addition of palmitic acid to medium containing lipid-free fetal calf serum did not elevate palmitic acid in cellular neutral lipids, while linoleic acid added to this medium increased the 18:2 acid level in this lipid fraction 5- to 10-fold.

Fatty Acid Composition of Cellular Phospholipids

The fatty acid composition of the total phospholipid fraction obtained from fetal calf serum, bovine serum, and HTC cells cultured on various levels of serum and lipid is given in Table IV. Serum phospholipid fatty acids differed from serum neutral lipid acids (Table III) in that they contained a higher percentage of the longer chain fatty acids. Fetal calf serum phospholipids contained a lower percentage of 18:2 and a higher percentage of C-22 and higher fatty acids than bovine serum phospholipids. Cellular phospholipid fatty acids showed more change when media serum and lipid levels were varied than did the neutral lipids. Palmitic and stearic acids remained relatively constant under all growth conditions, whereas 18:1 increased as serum and lipid levels were decreased. Cells grown on medium supplemented with lipid-free fetal calf serum contained the lowest level of 18:2 and 20:4 acids. The addition of palmitic acid to medium containing lipid-free fetal calf serum did not elevate palmitic acid in cellular phospholipids, but the addition of linoleic acid to the medium caused an increase in 18:2, 20:2, 20:4, and 22:5 acid percentages.

Fatty Acid Composition of Media Neutral Lipids

Table V shows the fatty acid composition of the neutral lipid fraction, derived from first half (only two) and last half incubation media, obtained from several different growth condi-

TABLE IV

Fatty Acid Composition of Total Phospholipid Derived from Serums and Minimal Deviation Hepatoma Cells Grown on Media Containing Various Amounts of Lipid

Serums and basal medium supplement	Fatty acid percentages[a,b]													
	16:0	16:1	18:0	18:1	18:2	20:1	20:2	20:3	20:4	22:2	24:0	24:1 + 22:4	22:5	22:6
Serums														
Fetal calf serum	20.9	0.8	20.2	19.8	1.5	0.5	---	2.5	8.4	T[c]	2.0	6.3	6.5	6.3
Bovine serum	18.3	0.8	25.5	16.0	12.7	T	---	5.9	6.9	1.3	1.2	3.2	3.3	1.1
Minimal deviation hepatoma														
20% Bovine serum + 5% fetal calf serum	15.0	3.3	14.9	31.1	7.5	1.5	T	1.8	15.0	1.0	1.3	2.0	2.1	1.6
10% Bovine serum + 5% fetal calf serum	16.2	3.4	18.2	39.6	9.6	T	T	---	11.6	T	T	1.4	T	T
5% Bovine serum + 5% fetal calf serum	14.9	3.7	16.3	36.3	7.6	1.7	T	2.0	8.6	1.0	1.4	2.7	1.3	1.6
5% Fetal calf serum	13.4	4.9	15.5	41.4	4.0	2.2	0.7	1.3	8.0	0.8	1.6	2.4	1.0	1.9
5% Bovine serum	9.5	3.1	16.9	44.5	9.7	1.9	---	---	12.7	T	T	T	T	T
2.5% Fetal calf serum	10.5	4.6	13.3	42.8	5.3	2.7	0.7	1.8	8.7	1.8	1.2	3.0	1.0	1.7
5% Lipid-free fetal calf serum	13.5	4.5	15.3	45.2	2.6	2.0	T	T	3.5	11.0	---	T	---	---
5% Lipid-free fetal calf serum + palmitic acid[d]	15.0	1.8	20.6	31.4	11.1	T	T	---	7.6	12.4	---	---	---	---
5% Lipid-free fetal calf serum + linoleic acid[d]	15.3	2.3	21.3	12.6	25.4	0.4	3.5	1.2	11.4	0.7	1.0	2.4	3.5	0.8

[a]Difference between the sum of the fatty acid percentages in each row and 100% represents the sum of minor components not given in the table.

[b]Percentages represent the mean of duplicate analyses on a single pooled sample.

[c]T = less than one half per cent.

[d]37.5 μg per ml of medium.

TABLE V

Comparison of Total Neutral Lipid Fatty Acid Composition of Serums and Media after HTC Cells were Cultured under Various Growth Conditions

Serums and basal medium supplement	Incubation period media were collected	Fatty acid percentages[a,b]							
		16:0	16:1	18:0	18:1	18:2	20:1	20:4	>20:4
Serums									
Fetal calf serum	—	23.5	7.1	4.5	33.8	9.3	0.9	12.5	6.4
Bovine serum	—	9.8	3.3	5.4	12.8	57.4	4.2	4.1	T[c]
Media									
20% Bovine serum + 5% fetal calf serum	Total	9.0	3.4	1.6	12.4	67.5	3.8	3.0	—
10% Bovine serum + 5% fetal calf serum	Last half	9.5	3.9	1.5	12.2	64.5	4.0	4.0	T
5% Bovine serum + 5% fetal calf serum	Last half	10.0	4.4	1.2	12.8	62.1	4.0	4.8	1.5
5% Fetal calf serum	Last half	24.4	10.3	3.4	31.4	11.2	0.7	9.8	3.4
5% Bovine serum	Last half	8.6	3.6	3.0	16.8	61.2	4.4	2.4	—
2.5% Fetal calf serum	First half	21.5	8.9	4.6	33.6	16.6	1.1	8.3	2.1
2.5% Fetal calf serum	Last half	17.2	4.3	7.1	51.0	6.8	—	7.8	5.9
5% Lipid-free fetal calf serum	First half	23.5	10.5	7.1	34.2	10.4	1.0	6.9	5.0
5% Lipid-free fetal calf serum	Last half	14.4	11.2	7.9	53.9	4.1	1.2	3.6	0.7
5% Lipid-free fetal calf serum + palmitic acid[d]	Last half	22.9	9.6	7.5	42.3	7.7	T	6.7	2.5
5% Lipid-free fetal calf serum + linoleic acid[d]	Last half	10.1	3.7	12.2	27.8	40.1	—	2.5	T

[a]Difference between the sum of the fatty acid percentages in each row and 100% represents the sum of minor components not given in the table.

[b]Percentages represent the mean of duplicate analyses on a single pooled sample.

[c]T = detectable but less than one half per cent.

[d]37.5 μg per ml of media.

tions. The percentage distribution of fatty acids from bovine and fetal calf serum neutral lipids also are given for comparison. When bovine serum at any concentration was used to supplement the media, 18:2 acid represented more than 60% of the neutral lipid fatty acids after incubation. Medium from cells grown on lipid-free fetal calf serum, plus linoleic, contained a high level of linoleic acid but not as high as when bovine serum was used in the culture media. The addition of palmitic acid to medium, supplemented with lipid-free fetal calf serum, did not elevate the concentration of palmitic in the medium after incubation. When either whole fetal calf serum or lipid-free fetal calf serum was used to supplement the medium, the second half incubation media contained higher levels of 18:1 acid and lower 18:2 acid concentrations than did the first half incubation media.

Fatty Acid Composition of Media Phospholipids

The fatty acid composition of the phospholipid fractions, derived from the last half incubation media obtained from the several different growth conditions, is given in Table VI. Media phospholipid fatty acids were distinctly different from media neutral lipid fatty acids (Table V). Stearic and 18:2 acid levels were noticeably higher when the media contained bovine serum, whereas those media supplemented with fetal calf serum or lipid-free fetal calf serum contained less stearate and higher percentages of 18:1 acid. Media, supplemented with only fetal calf serum or lipid-free fetal calf serum, contained relatively higher percentages of 16:1 acid, but bovine serum supplemented media did not contain measurable levels of this acid. The addition of palmitic acid and linoleic acid to media containing lipid-free fetal calf serum led to an increase in the concentrations of stearic and 18:2 acids, respectively, in media phospholipids after cell cultivation. Generally, the fatty acid composition of first and last half media phospholipids were similar.

DISCUSSION

A number of investigators (12,24-31) has

TABLE VI

Comparison of Total Phospholipid Fatty Acid Composition of Serums and Media after Minimal Deviation Hepatoma Cells were Cultured under Various Growth Conditions

Serums and basal medium supplement	Fatty acid percentages[a,b]									
	16:0	16:1	18:0	18:1	18:2	20:3	20:4	24:0	22:4 + 24:1	22:5
Serums										
Fetal calf serum	20.9	0.8	20.2	19.8	1.5	2.5	8.4	2.0	6.3	6.5
Bovine serum	18.3	0.8	25.5	16.0	12.7	5.9	6.9	1.2	3.2	3.3
Media[c]										
20% Bovine serum + 5% fetal calf serum	23.1	—	29.4	20.5	16.1	4.5	6.5	—	T[d]	T
10% Bovine serum + 5% fetal calf serum	22.4	—	31.4	21.4	12.2	5.2	6.5	—	T	T
5% Bovine serum + 5% fetal calf serum	26.8	—	30.3	22.4	10.3	4.2	5.9	—	T	T
5% Fetal calf serum	20.7	8.0	15.7	43.4	2.4	—	4.6	T	T	T
5% Bovine serum	15.8	—	26.3	21.4	13.4	6.1	7.6	T	T	1.8
2.5% Fetal calf serum	20.8	5.4	18.1	34.6	3.4	1.3	7.0	0.6	1.3	2.4
5% Lipid-free fetal calf serum	21.4	4.9	16.7	36.5	2.0	—	4.6	1.8	5.7	2.1
5% Lipid-free fetal calf serum + palmitic acid[e]	22.3	3.4	22.7	31.4	2.6	1.2	6.3	2.7	4.0	2.0
5% Lipid-free fetal calf serum + linoleic acid[e]	20.5	3.5	17.2	17.4	21.4	—	9.0	1.9	2.6	3.2

[a]Difference between the sum of the fatty acid percentages in each row and 100% represents the sum of components not given in the table.

[b]Percentages represent the mean of duplicate analyses on a single pooled sample.

[c]Media was from the last half of the incubation in all cases except when the medium was supplemented with 20% bovine serum plus 5% fetal calf serum, which was media for the total incubation time.

[d]T = detectable but less than one half per cent.

[e]37.5 μg per ml of medium.

shown, with a variety of cultured cells, that, when serum lipids were present in the media, little cellular lipids came from de novo biosynthesis and most were derived from the serum. Therefore, this investigation was initiated to determine the minimum level of serum lipids HTC cells require for growth and to determine what lipid biosynthesis this cell is capable of carrying out in the absence of or at low levels of exogenous serum lipid.

Cell Growth

Bovine serum contains ca. four times more lipid/ml than fetal calf serum. Therefore, the amount of lipid in the media was reduced more than 90% from the level of the stock cultures when it was determined that the modified Swim's 77 medium supplemented with 5% fetal calf serum supported good growth. This level of lipid (38.5 μg/ml medium) was ca. the same as that used by Watson (12) in growing suspension cultures of HTC cells. Medium containing 2.5% fetal calf serum did show some growth but probably would not have supported growth for any extended period of time. Stock cultures grown on 25% serum and used as starter cultures in all experiments probably contained enough stored lipids, growth factors, etc., to permit one or two cell divisions. Medium supplemented with 5% lipid-free fetal calf serum or supplemented with lipid-free fetal calf serum, plus either linoleic or palmitic acids, gave poor growth of HTC cells in roller cultures. However, the level of free acids used was probably inhibitory rather than growth promoting. Recent evidence from this laboratory (unpublished) has shown that lower levels of linoleic acid show some growth stimulating activity, while the higher levels prevent growth. Gerschenson, et al., (32) has shown that low levels of linoleic added to HeLa cells growing in lipid-deficient medium stimulated cell growth. Ham (33) also has suggested that linoleic acid was a growth factor for cultured Chinese hamster cells.

Extraction of Serum Lipids

Described procedures for reducing the amount of lipid in serum (12,34-36) either required too much time, low temperatures, and purification of the proteins precipitated with organic solvents, or failed to remove all the phospholipids. In search of a simple and quick method that would remove all the lipids, we tried chloroform-methanol (2:1) extraction of lyophilized serum. Surprisingly, the dry chloroform-methanol insoluble material dissolved completely in either distilled water or Swim's 77 medium and showed little denatured protein, as judged by filtration (0.2 μ pore size) and polyacrylamide gel electrophoresis (a single run). The lipids extracted from the serum with chloroform-methanol were chromatographically (GLC and TLC) identical to the lipids extracted from lyophilized fetal calf serum by the Bligh and Dyer procedure (19).

Media Lipids

The accumulation of both neutral lipids and phospholipids in the media under all growth conditions (Table II) was unexpected and apparently is the first report of lipid excretion by HTC cells. Hruban, et al., (37) has reported the formation and excretion of lipoproteins by a number of other hepatomas based upon histological examinations. The accumulation of lipids in the media of all growth conditions is of interest since most cultured cells studied thus far obtain a high percentage of their lipids from the media. The effect of exogenous lipids on lipid metabolism of cultured cells has been reviewed by Rothblat (38) and Bailey, et al. (39). Media neutral lipid and phospholipid profiles do not indicate a predominance of any one lipid class under any growth condition. Contrarily, the major lipid components from lipid-free serum supplemented medium, as well as the other media, were sterol esters, free fatty acids, free sterol, sphingomyelin, phosphatidylcholine, and an unidentified compound. The excretion of free sterols has been shown to occur in L-cells and MBIII cells by Bailey (40) who also reported data to suggest the excretion may be under the influence of serum globulins. Cultured L5178Y cells have been shown to increase the release of free sterol when phospholipids were added to the medium containing either delipidized serum protein or serum albumin (41). The excretion of other lipid classes by cultured cells does not appear to have been reported previously.

The similarity between the media neutral lipid and phospholipid class profiles and the corresponding pattern of the serums suggest that HTC cells may excrete into the media lipids with the same class composition as serum. Phosphatidylethanolamine and triglycerides are present in only trace amounts in serums, and they are also absent from all media even though the cells contain high levels of these two lipid classes. The presence of lipid classes in the cell, but not in the medium in which the cells were grown, is important. First, it rules against the possibility that the lipids, assumed to be excreted by the cells, did not arise from dead cells; and secondly, it suggests that the cellular lipids are not in equilibrium with media lipids. The reasons are numerous as to why the HTC cells may excrete lipids into the media of the same lipid class composition as serum. The most logical reason seems to be that these cells are performing functions similar to that carried out by normal liver cells. Another reason may be that serum lipids induce the synthesis of only the lipid classes present in the serum. A third possibility is that these cells may continue to secrete lipids into the medium in the presence of serum, because these cancer cells may have lost their feedback control. The loss of feedback control of cholesterol biosynthesis in almost all tumors is well known and has been reviewed recently by Sabine (42).

When low levels of fetal calf serum or lipid-free fetal calf serum were used to supplement the medium, significant differences in the fatty acid composition between first and last half media neutral lipids were observed. The composition of the media lipids from the last half of the incubation probably more nearly represented the composition of the fatty acids the cells excreted under the particular nutritional regime, because any cellular stores of lipid accumulated during the growth of the starter cultures would have been depleted or removed in the first half incubation media. This conclusion is supported by the fact that higher levels of 18:1 acid and lower levels of palmitic acid from the last half incubation media approach the percentage composition of these acids found in the cells. The difference in the fatty acid composition of the serum neutral lipids and phospholipids from media neutral lipids and phospholipids is significant. The higher percentages of monoenoic acids in the media, than were present originally in the serum lipid fractions, demonstrate that acyl moieties were excreted. The increased level of media 18:2 acid in the phospholipid fraction from cells grown on lipid-free fetal calf serum, plus linoleic acid, indicated the ability of the cells to take up free fatty acids, incorporate them into phospholipids, and excrete them into the medium. The ability of the HTC cell to carry out the above mentioned sequence of

events has been established independently in this laboratory using labeled palmitic acid.

Cellular Lipids

Cellular neutral lipid and phospholipid patterns (Figs. 1 and 2) were distinct from both serum and media lipid patterns. Although some differences were observed, a visual comparison of cellular lipid classes does not reveal the dramatic alteration in lipid patterns one might have expected as a result of the extremes in growth conditions. This suggests that exogenous serum lipids may have less influence on HTC cell lipid biosynthesis than previously reported for most other cultured cells. Triglyceride biosynthesis appeared to be inhibited by the absence of fetal calf serum or the presence of only bovine serum in the medium. Sterol ester synthesis appeared to be reduced by high levels of free fatty acids. The decreased level of triglycerides and sterol esters illustrates that cellular lipid turnover occurred.

Detectable quantities of glyceryl ether diesters were not observed in the HTC cells or media obtained from the various growth conditions. These data demonstrate that the presence of glyceryl ether diesters is not a characteristic of all neoplasms and, therefore, is not a good criterion for identifying neoplasms, as has been suggested previously (43,44). The phospholipids of HTC cells also contain low levels of plasmalogens, as judged by the small size of the aldehyde peaks when the methyl esters from the phospholipid fraction were analyzed.

The increased level of 20:2 and 20:4 acids indicates that the HTC cell can elongate and desaturate dienoic acids. Harary, et al., (45) has shown that HeLa cells and heart cells cultured for long periods of time show poor conversion of linoleic acid to arachidonic acid, whereas fresh heart cells performed this conversion readily.

The differences in the class compositions of the cells and the serums indicate that HTC cells are capable of synthesizing triglycerides, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol. The class composition of the cells grown on medium supplemented with lipid-free fetal calf serum further suggests that these cells also can synthesize sterol esters, free fatty acids, sterols, phosphatidylcholine, and sphingomyelin. Results from this laboratory, that will appear in a separate publication, show that radioactive labeled fatty acids are incorporated into individual phospholipids and acyl containing neutral lipid classes by these cells. These data suggest that HTC cells are capable of synthesizing most, if not all, of their structural neutral lipids and phosphoglycerides.

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REFERENCES

1. "Biological Membranes," Edited by D. Chapman, Academic Press, New York, N.Y., 1968.
2. "Structure and Function of Biological Membranes," Edited by L.I. Rothfield, Academic Press, New York, N.Y., 1971.
3. Korn, E.D., Annu. Rev. Biochem. 38:263 (1969).
4. Rothfield, L., and D. Romeo in "Structure and Function of Biological Membranes," Edited by L.I. Rothfield, Academic Press, New York, 1971, p. 231.
5. Bergstrom, S., L.A. Carlson, and J.R. Weeks, Pharmacol. Rev. 20:1 (1968).
6. Horton, E.W., Physiol. Rev. 49:122 (1969).
7. Cotmore, J.M., G. Nichols, Jr., and R.E. Wuthier, Science 172:1339 (1971).
8. Hegyvary, C., and R.L. Post in "The Molecular Basis of Membrane Function," Edited by D.C. Tosteson, Prentice-Hall, Inc., Englewood Cliffs, N.J., p. 519 (1969).
9. Kahlenberg, A., and B. Banjo, J. Biol. Chem. 247:1156 (1972).
10. Tumor Lipids: Biochemistry and Metabolism, Edited by Randall Wood, American Oil Chemists' Society, Champaign, Ill. 1973.
11. Morris, H.P., H. Sidransky, B.P. Wagner and H.M. Dyer, Cancer Res. 20:1252 (1960).
12. Watson, J.A., Lipids 7:146 (1972).
13. Brady, R.O., C. Borck, and R.M. Bradley, J. Biol. Chem. 244:6552 (1969).
14. Ontko, J.A., J. Biol. Chem. 247:1788 (1972).
15. Goodridge, A.G., Ibid. 248:1924 (1973).
16. Thompson, E.B., G.M. Tomkins and J.F. Curran, Natl. Acad. Sci. Proc. 56:296 (1966).
17. Nowell, P.C., H.P. Morris and Van R. Potter, Cancer Res. 27:1565 (1967).
18. Tomkins, G.M., E.B. Thomson, S. Hayashi, T. Gelehrter, D. Grannes and B. Peterkofsky, Quant. Biol. 31:399 (1966).
19. Bligh, E.G., and W.J. Dyer, Can. J. Biochem. Physiol. 37:911 (1959).
20. Bergstrom, B., Acta Physiol. Scand. 25:101 (1952).
21. Rouser, G., A.M. Siakotos, and S. Fleischer, Lipids 1:85 (1966).
22. Dittmer, J.C., and R.L. Lester, J. Lipid Res. 5:126 (1964).
23. Wood, R., Lipids 7:596 (1972).
24. Howard, B.V., and D. Kritchevsky, Biochim. Biophys. Acta 187:293 (1969).
25. Bailey, J.M., Ibid. 125:226 (1966).
26. MacKenzie, C.G., J.B. MacKenzie, O.K. Reiss and J.A. Wisneski, J. Lipid Res. 11:571 (1970).
27. MacKenzie, C.G., J.B. MacKenzie and O.K. Reiss, "In Lipid Metabolism in Tissue Culture Cells," Edited by G.H. Rothblat and D. Kritchevsky, Wistar Institute Symposium Monograph, 6, 1967, p. 63.
28. Moskowitz, M.S., Ibid. p. 49.
29. Geyer, R.P., Ibid. p. 33.
30. Bailey, J.M., G.O. Gey and M.K. Gey, Proc. Soc. Exp. Biol. Med. 100:686 (1959).
31. Jacobs, R.A., W.S. Sly and P.W. Majerus, J. Biol. Chem. 248:1268 (1973).
32. Gerschenson, L.E., J.F. Mead, I. Harary and D.F. Haggerty, Jr., Biochim. Biophys. Acta 131:42

(1967).
33. Ham, R.G., Science 140:802 (1963).
34. Albutt, E.C., J. Med. Lab. Tech., 23:61 (1966).
35. Williams, C.D., and J. Avigan, Biochim. Biophys. Acta, 260:413 (1972).
36. Avigan, J., J. Biol. Chem. 226:957 (1957).
37. Hruban, Z., Y. Mochizuke, Morris, H.P. and A. Slesers, Lab. Invest. 26:86 (1972).
38. Rothblat, G.H., Advan. Lipid Res. 7:135 (1969).
39. Bailey, J.M., B.V. Howard, L.M. Dunbar and S.F. Tillman, Lipids 7:125 (1972).
40. Bailey, J.M., in "Lipid Metabolism in Tissue Culture Cells," Edited by G.H. Rothblat and D. Kritchevsky, Wistar Institute Symposium Monograph, 6, 1967, p. 85.
41. Burns, C.H., and G.H. Rothblat, Biochim. Biophys. Acta. 176:616 (1969).
42. Sabine, J.R., in "Tumor Lipids: Biochemistry and Metabolism," Edited by Randall Wood, American Oil Chemists' Society, Champaign, Ill. 1973.
43. Snyder, F., and R. Wood, Cancer Res. 29:251 (1969).
44. Snyder, F., and R. Wood, Ibid. 28:972 (1968).
45. Harary, I., L.E. Gerschenson, D.F. Haggerty, Jr., W. Desmond and J.F. Mead, in "Lipid Metabolism in Tissue Culture Cells," Edited by G.H. Rothblat and D. Kritchevsky, Wistar Institute Symposium Monograph 6, 1967, p. 17.

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Lipids of Cultured Hepatoma Cells: II. Effect of Media Lipids on Cellular Phospholipids

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ABSTRACT

Minimal deviation hepatoma cells were cultured in a modified Swim's 77 medium supplemented with decreasing amounts of serum, lipid-free serum, and lipid-free serum containing added palmitic or linoleic acids. Cellular phospholipids were extracted and the class distribution determined quantitatively. The fatty acid composition of each phospholipid class was determined, and the percentages from cells grown on each of the various media were compared. Cellular phospholipid class and fatty acid compositions differed from media compositions, indicating that intact serum phospholipids are not incorporated into cellular structures. Phosphatidylcholine percentages decreased as the media serum and lipid levels decreased, while phosphatidylinositol and phosphatidylethanolamine percentages increased. Sphingomyelin of cells grown in medium containing added linoleic acids contained a high level of a 24:2 acid. All classes, except sphingomyelin, contained elevated levels of 18:1 acid and decreased levels of polyunsaturated fatty acids, relative to normal rat liver. Cells cultured on lipid-free medium did not contain increased concentrations of 20:3 acid, suggesting that this hepatoma cell cannot desaturate monoenoic acids. Phosphoglycerides of cells, grown on lipid-free medium, had the highest monoene fatty acid concentration, whereas those cells grown on media containing added linoleic acid had the lowest concentrations, suggesting that linoleate may inhibit or regulate monoenoic acid biosynthesis in this cell. These mass data also demonstrate that mono-

TABLE I

Phospholipid Percentages of Minimal Deviation Hepatoma Cells Grown in Culture on Media Containing Varying Amounts of Lipid[a]

Basal medium supplement	Percentages[b]									
	Orig.	Lyso-PC	SPH	PC	PI	PS	X_1	PE	X_2	SF[c]
20% Bovine serum + 5% fetal calf serum	0.6	1.7	8.8	41.4	8.4	6.6	—	26.4	2.0	4.4
10% Bovine serum + 5% fetal calf serum	0.4	1.1	5.9	35.4	11.8	7.4	—	31.5	2.0	4.3
5% Bovine serum + 5% fetal calf serum	0.6	1.6	8.9	34.8	10.5	6.2	—	31.3	2.0	4.1
5% Fetal calf serum	0.5	1.2	7.4	32.8	11.8	6.1	—	33.8	2.2	4.2
5% Bovine serum	0.8	1.4	7.5	28.8	19.3		—	35.9	1.7	4.6
2.5% Fetal calf serum	0.6	2.3	7.4	29.1	12.8	5.7	1.6	33.9	1.8	4.8
5% Lipid-free fetal calf serum	1.0	3.0	7.9	27.1	11.6	6.0	1.8	34.8	1.8	5.0
5% Lipid-free fetal calf serum + palmitic acid[d]	6.9	3.4	7.6	27.9	14.3	1.7	—	30.3	3.4	4.5
5% Lipid-free fetal calf serum + linoleic acid[d]	1.1	3.6	12.0	26.1	14.2	5.1	—	28.3	3.8	5.9
Fetal calf serum	1.2	21.7	25.1	50.2	—	—	—	—	—	2.0
Bovine serum	0.7	10.8	18.3	67.0	—	—	—	—	—	0.9

[a]Orig. = origin, Lyso-PC = lyso-phosphatidylcholine, SPH = sphingomyelin, PC = phosphatidylcholine, PI = phosphatidylinositol, PS = phosphatidylserine, X_1 = unidentified phosphorous containing compound migrating between phosphatidylserine and phosphatidylethanolamine, PE = phosphatidylethanolamine, X_2 = unidentified phosphorous containing compound migrating between phosphatidylethanolamine and the solvent front, and SF = solvent front.

[b]Percentages represent the mean of duplicate phosphorous analyses, and values have not been corrected for differences in mol wt of lipid classes.

[c]Solvent front material consists primarily of diphosphatidylglycerol but also contains minor amounts of other phosphorous and nonphosphorous containing compounds.

[d]37.5 μg/ml of medium.

enoic fatty acid biosynthesis in this cultured hepatoma cell responds to dietary changes.

INTRODUCTION

The importance of lipids in many biological processes and the advantages of using minimal deviation hepatoma cells (HTC), grown in tissue culture, to study the structure and metabolism of lipids to determine their possible direct or indirect involvement in neoplasia have been discussed previously (1). To the authors' knowledge, the phospholipids of HTC cells grown in tissue culture have not been studied in any detail. Results from this laboratory (1) have shown that phospholipids of HTC cells, cultured in media containing various amounts of serum and lipids, showed little qualitative difference in class distributions and the same approximate total phospholipid fatty acid compositions.

The quantitative determination of phospholipid class compositions and fatty acid compositions of individual phosphoglycerides derived from HTC cells, grown on media containing varying levels of serum and lipids, is reported in this paper.

MATERIALS AND METHODS

Minimal deviation hepatoma 7288C cells were grown in roller cultures on a modified Swim's 77 medium supplemented with various levels of serums and lipids (see Table I for supplements), as described previously (1). Cells were harvested, lyophilized, extracted, and the lipids fractionated into neutral lipids and phospholipids as described earlier (1). Phospholipid classes were resolved by thin layer chromatography (TLC) on adsorbent layers of Silica Gel HR, developed in a solvent system of chloroform-methanol-acetic acid-0.9% saline, 50:25:8:4, v/v. Quantitation of individual classes resolved by TLC was determined by phosphorous analysis, according to the procedure of Rouser et al. (2). Chromatoplates were sprayed with sulfuric acid and charred for visualization and documentation by photography. Class identification was based upon the use of phosphorous and ninhydrin spray reagents (3), gas liquid chromatography (GLC) of methyl esters, and cochromatography with standards. Methyl esters of the phospholipids were prepared by acid catalyzed esterification and analyzed by GLC, as previously described (4). Identification of methyl esters is based upon relative retention times before and after hydrogenation and cochromatography with commercially available esters of known structure. Except for 24:0 and 24:1 acids, acids with retention times longer than 20:4 must be considered as tentatively identified, since there are a large number of esters with similar retention times possible in this area of the chromatogram. The use of classical names for unsaturated fatty esters does not imply that the double bond positions and configurations have been determined.

RESULTS

Phospholipid Distribution

The percentage distribution of the phospholipids, derived from HTC cells cultured on various media and from serums, is given in Table I. Serums, the only media component containing lipids, were characterized by high levels of lyso-phosphatidylcholine, sphingomyelin, and phosphatidylcholine, which differed dramatically from the cellular level of these phospholipids. Cellular phosphatidylcholine levels decreased significantly as the level of serum decreased to ca. 5%. The decrease of phosphatidylcholine was offset by increases in the concentrations of phosphatidylinositol and phosphatidylethanolamine. Sphingomyelin, phosphatidylserine, and other minor components showed little if any change as the lipid level of the media changed. Except for an increased level of sphingomyelin, when linoleic acid plus lipid-free fetal calf serum supplemented the medium, the addition of fatty acids to the medium had little effect upon the phospholipid composition relative to that of the cells grown in lipid-free medium.

Phosphatidylcholine

This lipid class, one of the major components of cells and serums, showed a percentage change in some of the fatty acids present in this phospholipid, as the serum and lipid levels of the media changed (Table II). The fatty acid compositions of cellular and serum phosphatidylcholines were different. Except where fatty acids were added to the media, palmitic and stearic acid percentages remained relatively constant, whereas 18:1 and 20:1 acids increased and 18:2 and 20:4 acids decreased with a decrease in media serum and lipid levels. Table II shows that linoleic acid, added to medium containing lipid-free fetal calf serum, increased cellular 18:2 and 20:4 acid levels, whereas added palmitic acid did not elevate phosphatidylcholine palmitate concentrations.

Phosphatidylethanolamine

The fatty acid compositions of phospha-

TABLE II

Comparison of Fatty Acid Composition of Phosphatidylcholine Derived from Minimal Deviation Hepatoma Cells Cultured in Media Containing Varying Amounts of Serum and Lipid

Basal medium supplement	Fatty acid percentages[a,b]								
	16:0	16:1	18:0	18:1	18:2	20:1	20:3	20:4	22:2
20% Bovine serum + 5% fetal calf serum	27.8	4.4	9.7	30.8	9.0	1.6	1.5	12.2	1.2
10% Bovine serum + 5% fetal calf serum	25.4	4.5	13.1	34.0	10.2	1.9	0.9	6.8	1.0
5% Bovine serum + 5% fetal calf serum	28.6	7.0	8.4	38.6	8.8	1.7	0.9	4.2	0.9
5% Fetal calf serum	28.0	7.3	9.4	44.5	3.7	2.4	T[c]	3.2	0.6
5% Bovine serum	20.6	6.8	8.1	39.6	9.6	2.6	1.2	7.2	0.9
2.5% Fetal calf serum	23.1	8.5	6.5	46.8	5.4	2.8	0.9	3.8	1.1
5% Lipid-free fetal calf serum	20.5	8.6	8.3	52.8	3.5	3.4	T	1.7	T
5% Lipid-free fetal calf serum + palmitic acid[d]	25.0	5.0	12.4	41.8	6.7	1.8	0.6	5.0	1.2
5% Lipid-free fetal calf serum + linoleic acid[d,e]	28.3	2.8	14.8	11.4	26.5	T	T	10.6	T
Bovine serum[f]	15.3	0.9	26.6	19.2	14.7	—	5.9	7.1	—
Fetal calf serum[g]	22.2	1.1	23.9	26.4	1.2	—	2.8	7.8	—

[a]Difference between sum of fatty acid percentages in each row and 100% represents sum of minor components not given in the table.

[b]Percentages represent the mean of duplicate analyses.

[c]T = detectable levels but less than one half per cent.

[d]37.5 μg/ml of medium.

[e]Contained 3.6% C-20:2 fatty acid.

[f]Sample also contained 2.9 and 0.5% of 22:5 and 22:6 respectively.

[g]Sample also contained 6.6 and 4.9% of 22:5 and 22:6 respectively.

tidylethanolamine, a major cellular phospholipid but absent from the serums, are given in Table III. This phospholipid class contained more stearic and less palmitic acids than phosphatidylcholine; but, as with phosphatidylcholine, the concentration of both of these acids remained relatively constant as serum lipid levels decreased. The percentages of 18:1 increased and 20:4 decreased as serum and lipid levels of the media decreased. The percentages of acids in phosphatidylethanolamine with retention times longer than 20:4 were too small to attach any significance to the observed changes in these acids. The addition of linoleic acid to the medium resulted in increased levels of 18:0, 18:2, and 20:4 acids, accompanied by a decrease in 18:1 levels, whereas the addition of palmitic acid to the medium caused only a rise in stearic acid levels but not palmitic.

Phosphatidylinositol and Phosphatidylserine

The fatty acid composition of phosphatidylinositol, phosphatidylserine, and, in some cases, of these two classes combined that was derived from HTC cells grown on various media is shown in Table IV. These cellular phosphatides, absent from the serums, were characterized by the highest levels of stearic acid of all the phosphatides. Phosphatidylinositol contained higher percentages of 18:0 and 20:4 and lower percentages of 18:1 than phosphatidylserine. Decreased serum and lipids in the media resulted in increased levels of 18:1 and decreased levels of 20:4 for both phosphatide classes. Phosphatidylserine contained higher percentages of acids with retention times longer than 20:4 than did phosphatidylinositol, but the values were too low to allow much significance to be attached to this observation. The level of 18:2 in these two lipid classes was generally low; however, when linoleic acid was added to the medium, 18:2 and 20:4 values increased at the expense of 18:1. Fatty acids added to the media did not alter palmitate and stearate levels of these combined phosphatide fractions.

Diphosphatidylglycerol

When the analytical TLCs were developed for phosphorous analysis (Table I), diphosphatidylglycerol was not resolved sufficiently from the solvent front to permit separate quantification; but, when preparative TLC was used to isolate individual phospholipid classes, a reasonably pure diphosphatidylglycerol fraction was obtained in most cases. The fatty acid composition of the diphosphatidylglycerol frac-

TABLE III

Comparison of Fatty Acid Composition of Phosphatidylethanolamine Derived from Minimal Deviation Hepatoma Cells Cultured in Media Containing Varying Amounts of Serum and Lipid

Basal medium supplement	Fatty acid percentages[a,b]										
	16:0	16:1	18:0	18:1	18:2	20:1	20:3	20:4	22:2	22:5	22:6
20% Bovine serum + 5% fetal calf serum	5.2	2.1	17.6	39.1	4.5	1.9	1.4	20.1	0.9	2.8	2.6
10% Bovine serum + 5% fetal calf serum	7.0	2.3	18.6	42.7	7.7	1.7	T[c]	14.5	0.7	1.6	1.5
5% Bovine serum + 5% fetal calf serum	7.5	2.9	18.2	44.2	7.3	1.5	1.6	12.4	1.3	1.2	1.6
5% Fetal calf serum	7.2	3.5	19.1	49.2	3.4	1.9	0.8	9.6	0.8	0.8	1.9
5% Bovine serum	5.4	2.7	17.4	45.9	8.3	2.9	0.9	10.4	0.7	2.1	0.8
2.5% Fetal calf serum	5.6	3.4	14.0	52.4	5.2	2.8	1.1	10.6	1.4	0.9	2.2
5% Lipid-free fetal calf serum	5.1	4.3	15.3	56.9	3.9	2.9	0.8	6.3	0.5	2.1	T
5% Lipid-free fetal calf serum + palmitic acid[d]	5.5	2.5	22.3	47.8	4.9	1.8	0.8	9.0	1.1	1.3	1.8
5% Lipid-free fetal calf serum + linoleic acid[d,e]	6.6	1.0	28.5	14.6	21.6	0.5	1.2	15.8	T	2.0	1.2

[a]Difference between sum of fatty acid percentages in each row and 100% represents the sum of minor components not given in the table.

[b]Percentages represent the mean of duplicate analyses.

[c]T = detectable levels but less than one half per cent.

[d]Contained 5.2% C-20:2 fatty acid.

[e]37.5 μg/ml of medium.

tion, obtained from cells grown on various media, is given in Table V. This class was characterized by low levels of stearate and palmitate and higher concentrations of 14:0, 16:1, 18:1, and 18:2 acids than found in other phosphatides. The concentration of monoenoic acids increased, while 18:2 levels decreased, as media serum lipid levels decreased. Palmitic acid added to medium containing lipid-free fetal calf serum had little influence on the composition of diphosphatidylglycerol, whereas added linoleic acid greatly elevated 18:2 percentages, while 18:1 levels decreased.

Sphingomyelin

The fatty acid compositions of sphingomyelin, derived from HTC cells grown on various media and from bovine and fetal calf serums, are shown in Table VI. Bovine serum sphingomyelin contained a high percentage of 16:0 and 23:0 acids, whereas fetal calf serum sphingomyelin contained a high percentage of 22:0 and 24:1 acids. The compositions of the serums differed from the sphingomyelin composition of the cells. Although some differences in sphingomyelin fatty acid composition were observed, they did not appear to be related to type of serum or amount of lipid in the medium. Palmitic acid, added to medium containing lipid-free fetal calf serum, did not elevate sphingomyelin palmitate but caused an increase in 18:0 and 24:0 percentages. Cells grown on medium containing added linoleic acid did not contain significant levels of 18:2 in the sphingomyelin acids but contained a high level of 24:2 acid.

Unidentified Material

A compound of the cellular phospholipids, absent from the serums, having an R_f value between phosphatidylethanolamine and diphosphatidylglycerol and representing ca. 2% of the phospholipid phosphorous was analyzed for fatty acids. The fatty acid composition of this compound differed from the other phospholipid classes. The major acids were 16:0, 18:0, and 18:1. The monoenoic acid peaks were broadened, indicating the presence of more than one component, but the small sample size prevented further characterization. Variability of the major fatty acid percentage seemed to be unrelated to serum and lipid levels, except when linoleic acid was added to the medium.

Under preparative TLC conditions, material migrating near the solvent front usually was resolved from diphosphatidylglycerol. The fatty acid composition of the solvent front material, containing a high level of 24:0 and 24:1 acids, suggests that ceramide was a major component. However, elevated levels of 18:1, relative to the percentage found in sphingomyelin, indicated the presence of other components. Except when linoleic acid was added to the growth medium, identifiable changes in the fatty acid

TABLE IV

Comparison of Fatty Acid Composition of Phosphatidylinositol and Phosphatidylserine Derived from Minimal Deviation Hepatoma Cells Cultured in Media Containing Varying Amounts of Serum and Lipid

Basal medium supplement	Lipid class[c]	Fatty acid percentages[a,b]										
		16:0	16:1	18:0	18:1	18:2	20:0	20:1	20:3	20:4	22:5	22:6
20% Bovine serum +	PI	1.5	0.4	49.5	23.9	3.2	0.3	0.7	2.3	15.0	1.2	0.5
5% fetal calf serum	PS	3.8	0.9	32.4	39.0	1.6	1.4	2.1	2.8	6.7	3.5	2.1
10% Bovine serum +	PI	2.4	1.3	46.5	26.1	3.8	T[d]	0.8	1.8	13.4	1.9	0.8
5% fetal calf serum	PS	3.5	1.2	34.3	40.6	2.7	1.4	2.1	1.6	6.4	1.9	1.3
5% Bovine serum +	PI	2.8	2.2	44.7	28.6	3.5	—	1.4	3.0	9.0	0.9	0.5
5% fetal calf serum	PS	3.1	1.4	36.3	40.6	3.1	1.1	2.2	2.4	4.8	1.1	1.4
5% Fetal calf serum	PI	3.4	2.3	47.2	31.7	1.4	0.2	1.3	0.9	7.3	0.7	0.8
	PS	4.4	1.7	37.8	44.2	1.2	1.3	1.9	1.4	1.4	1.5	1.7
5% Bovine serum	PI + PS	3.5	2.4	37.8	36.7	4.9	0.5	1.7	0.7	7.3	0.9	1.5
2.5% Fetal calf serum	PI	3.8	3.8	36.6	35.4	2.6	0.3	1.9	1.6	9.7	0.7	0.2
	PS	4.5	1.4	31.1	48.6	2.0	0.8	3.7	0.4	3.1	0.5	2.0
5% Lipid-free fetal calf serum	PI + PS	3.1	3.4	40.4	40.3	1.5	—	2.2	0.7	4.7	1.0	T
5% Lipid-free fetal calf serum + palmitic acid[e]	PI + PS	2.4	1.9	42.6	30.2	2.3	—	1.3	1.3	15.9	1.4	—
5% Lipid free-fetal calf serum + linoleic acid[e]	PI + PS	4.5	0.6	43.4	11.9	15.9	—	—	—	18.3	—	—

[a]Difference between sum of fatty acid percentages in each row and 100% represents the sum of minor components not given in the table.

[b]Percentages represent the mean of duplicate analyses.

[c]PI = phosphatidylinositol and PS = phosphatidylserine.

[d]T = detectable levels but less than one half per cent.

[e]37.5 μg/ml of medium.

composition of this fraction did not appear to be related to levels of serum and lipids in the media.

DISCUSSION

The class compositional data of the phospholipids, derived from HTC cells grown on various media, demonstrate that the percentages of some phosphoglycerides can be affected by media serum and lipid levels, while others are unaffected. The change is not restricted to the lipids found in media since phosphatidylethanolamine and phosphatidylinositol, absent from bovine and fetal calf serum, were two of three classes showing the most change. These data indicate that care must be exercised when comparing phospholipid data, even from the same cell line, with that obtained in other laboratories, especially when the growth conditions are not known.

The phospholipid class compositions of the HTC cells grown on various media did not agree with compositions reported for rat liver (5-7); however, the composition of the cells cultured on 25% serum more nearly agreed. Even when the media contained high levels of serum, phosphatidylcholine, the major serum phospholipid, was lower in HTC cells than rat liver; and phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol, absent from serum, were elevated above rat liver percentages. Bergelson et al. (5) have shown that host grown hepatoma 27 contained elevated levels of phosphatidylserine, phosphatidylethanolamine, and sphingomyelin and a lower level of phosphatidylcholine, relative to rat liver values. Morris hepatoma 3924A, a fast growing hepatoma, was shown to contain decreased levels of phosphatidylcholine and higher amounts of phosphatidylinositol (8). The same investigators also reported that the level of phosphatidylcholine in the Ruber H-35 hepatoma was lower than rat liver levels; however, the latter analysis was complicated by a high percentage of a polar phosphorous containing compound. These comparisons indicate that cultured HTC cells have an altered phospholipid composition, relative to rat liver, similar in some respects to solid hepatomas grown in host animals.

Each phospholipid class from HTC cells, like that of liver, exhibited a characteristic fatty acid composition that changed with changes in media serum and lipid levels. Except for sphingomyelin, phosphatidylcholine had the highest level of palmitate. Phosphatidylcholine of the cultured cells contained reduced levels of 18:0, 20:4, and 22:6 acids and a much higher concentration of 18:1, relative to values reported for rat liver lecithin (9-12). Increased concentrations of oleic acid in phosphatidyl-

TABLE V

Comparison of Fatty Acid Composition of Diphosphatidylglycerol Derived from Minimal Deviation Hepatoma Cells Cultured in Media Containing Varying Amounts of Serum and Lipid

Basal medium supplement	Fatty acid percentages[a,b]										
	14:0	16:0	16:1	18:0	18:1	18:2	20:1	20:3	20:4	24:0	22:4 + 24:1	22:4 + 24:1
20% Bovine serum + 5% fetal calf serum	0.5	8.3	9.6	2.5	44.7	23.4	0.5	1.6	1.8	1.4	1.6	—
10% Bovine serum + 5% fetal calf serum	1.2	8.5	9.5	2.7	44.7	27.1	0.7	1.1	0.6	1.6	1.4	—
5% Bovine serum + 5% fetal calf serum	1.0	6.7	13.6	1.8	43.8	27.6	—	1.7	T[c]	1.5	1.4	—
5% Fetal calf serum[d]	2.2	14.7	12.1	8.0	43.4	4.7	2.0	T	T	2.1	1.8	—
5% Bovine serum	2.1	8.5	10.5	3.6	46.9	19.1	1.1	0.8	1.5	0.7	1.7	1.8
2.5% Fetal calf serum	1.5	6.5	14.8	1.6	53.6	15.2	0.6	0.7	0.5	1.4	2.6	—
5% Lipid-free fetal calf serum	1.4	6.1	16.2	2.1	59.5	8.6	1.9	T	1.0	0.8	1.2	1.3
5% Lipid-free fetal calf serum + palmitic acid[d,e]	2.2	9.8	9.3	4.7	49.2	12.8	1.3	1.0	2.1	1.7	T	T
5% Lipid-free fetal calf serum + linoleic acid[e]	2.1	9.9	4.1	4.5	12.7	51.7	0.6	1.7	6.1	T	1.3	1.1

[a]Difference between sum of fatty acid percentages in each row and 100% represents the sum of minor components not given in the table.

[b]Percentages represent the mean of duplicate analyses.

[c]T = detectable levels but less than one half per cent.

[d]Also contained solvent front fraction that was not resolved from diphosphatidylglycerol in this sample.

[e]37.5 μg/ml of medium.

TABLE VI

Comparison of Fatty Acid Composition of Sphingomyelin Derived from Minimal Deviation Hepatoma Cells Cultured in Media Containing Varying Amounts of Serum and Lipid

Basal medium supplement	Fatty acid percentages[a,b]								
	16:0	18:0	18:1	22:0	22:1	23:0	24:0	24:1	24:2
20% Bovine serum + 5% fetal calf serum	30.8	6.1	1.1	9.4	1.4	4.3	23.6	20.2	T[c]
10% Bovine serum + 5% fetal calf serum	27.0	6.3	2.2	8.9	0.5	2.7	22.9	27.6	T
5% Bovine serum + 5% fetal calf serum	39.2	5.9	1.3	7.2	1.6	1.5	18.3	23.2	—
5% Fetal calf serum	35.3	7.1	1.7	6.9	0.9	1.0	24.4	21.6	—
5% Bovine serum	28.4	4.7	3.3	5.6	2.6	2.2	13.7	34.3	—
2.5% Fetal calf serum	33.0	4.5	2.8	5.9	2.8	1.0	17.0	31.5	—
5% Lipid-free fetal calf serum	30.7	5.6	3.6	5.8	3.7	0.6	17.4	30.5	—
5% Lipid-free fetal calf serum + palmitic acid[d]	28.7	11.2	4.3	7.3	T	—	26.0	21.6	—
5% Lipid-free fetal calf serum + linoleic acid[d]	30.3	7.2	1.2	4.7	—	—	10.2	25.7	23.0
Bovine serum	47.3	11.8	1.3	6.4	—	12.8	9.6	7.5	0.5
Fetal calf serum[e]	29.1	10.6	1.2	15.9	1.5	2.4	9.8	23.8	1.2

[a]Difference between sum of fatty acid percentages in each row and 100% represents the sum of minor components not given in the table.

[b]Percentages represent the mean of duplicate analyses.

[c]T = detectable levels but less than one half per cent.

[d]37.5 μg/ml of medium.

[e]Sample contained 2.6% of 20:0.

choline from hepatomas and hepatoma cell fractions have been reported previously by Ruggieri and Fallani (13), Bergelson and Dyatlovitskaya (14), and van Hoeven and Emmelot (15).

Rat liver phosphatidylethanolamine usually contains 40-50% saturated acids (9,11,12), but the saturated acids of the HTC cells, particularly palmitic acid, were reduced greatly. This class had the highest levels of 20:4 and 22:6, but the values were much lower than percentages of these acids in rat liver phosphatidylethanolamine (9,11,12). The decreased levels of polyunsaturated acids and palmitic acid were replaced with oleic acid. van Hoeven and Emmelot (15) have shown that phosphatidylethanolamine derived from hepatoma 484A plasma membranes had a much lower stearic/oleic ratio than normal membranes, but they did not see a decreased ratio in two mouse hepatomas, nor did Ruggieri and Fallani (16) observe a significant increase in oleic acid of hepatoma 5123 phosphatidylethanolamine.

Phosphatidylserine and phosphatidylinositol were characterized by a high level of stearic acid, which is typical of rat liver (11,17). The significant reductions in the levels of 20:4 in phosphatidylinositol and 20:4 and 22:6 in phosphatidylserine, relative to the concentrations found in these phospholipid classes of rat liver (17), are compensated for by increased oleic acid levels. Both of these phosphatides, isolated from plasma membranes of a rat hepatoma, have been shown to contain a decreased stearic/oleic acid ratio, but two mouse hepatomas did not (15).

Diphosphatidylglycerol contained the highest level of 18:2, but the concentrations were reduced greatly from the levels found in rat liver (17). The lower levels of 18:2 were offset by increased percentages of both 16:1 and 18:1. It is easy to see how a number of mitochondrial enzymes that require diphosphatidylglycerol for activity could be affected by a change in the molecular species of this class.

The characteristic composition of rat liver sphingomyelin (17) also prevailed in the HTC cells, except for a somewhat higher level of palmitic acid. This was the only phospholipid class of HTC cells that did not show an elevated level of oleic. It is of interest that, when linoleic acid was added to the medium, a very high percentage of 24:2 appeared, while only traces of this acid were found when cells were grown on media containing 20% bovine serum, which contained twice the level of esterified linoleic acid. We have shown previously (18) that sphingomyelin from Ehrlich ascites cells contained a high level of this acid, which is absent, or present in only trace amounts, in normal mature tissue. The enzyme system responsible for the synthesis of 24:0 and 24:1 may have

lost some of its specificity in neoplasms and, therefore, is able to accept and elongate linoleic acid. The presence of this long chain dienoic acid in the glycosphingolipids of the surface membranes could have far-reaching effect upon the cell.

Generally, all phospholipid classes, except sphingomyelin, showed a decrease in polyunsaturated acids, especially 20:4, and an increase in monoenoic acids, particularly oleic acid, as the serum and lipid levels decreased. The decreased cellular levels of 18:2 and 20:4 acids were expected, since mammalian cells require exogenous sources of these acids, but the replacement of these acids with only monoenoic acids was unexpected. Bailey et al. (19) have shown that an increase in the levels of 16:1, 18:1, 20:3, and, in some instances, 22:3 occurred when several cell lines were grown under conditions where an essential fatty acid deficiency existed. The absence of increased levels of 20:3 or 22:3 in either the neutral lipids (1) or phospholipids, even when the cells were cultured on lipid-free medium, suggests that these cells may not be able to desaturate monoene fatty acids further. We have shown in a separate publication (20) that these cells can desaturate saturated acids to monoenes, and the data in this study suggest that, to a limited extent, linoleic acid can be converted into arachidonic acid.

The concentration of oleic acid in all the phosphatides was the highest when the cells were grown on lipid-free medium and was the lowest when the cells were grown on medium containing added free linoleic acid or 25% serum. The possibility that free fatty acids, which also were present in serum, might have caused the decrease in the cellular concentrations of oleic acid is negated by the fact that palmitic acid added to the medium had little effect on oleic acid levels. Another factor common to both media was the high concentrations of linoleic acid: ca. 68 μg/ml of the 20% bovine serum-5% fetal calf serum medium and 37.5 μg/ml of the medium containing added linoleic acid. The much greater effect of the free linoleate probably is related to its greater availability to the cells. These data suggest that the level of linoleic acid may inhibit or regulate monoenoic acid biosynthesis in these cultured rat hepatoma cells. The influence on monoenoic acid may be only apparent and actually may affect all endogenously biosynthesized fatty acids. It has been shown previously (21-23) that mouse hepatic lipogenesis, as measured by the rate of ^{14}C-acetate incorporation into lipid, is influenced more by dietary linoleate than other fatty acids. Unlike the mouse, rat liver hepatic fatty acid biosynthesis is less sensitive to dietary lipids; however, recent evidence indicates that dietary safflower oil (trilinolein) also inhibits liver fatty acid synthesis in this species (23). The decreased fatty acid biosynthesis in cultured cells, when serum is added (19,24) to the medium, may be due to linoleate in the serum. Recent work by Jacobs et al. (25), with cultured primary skin fibroblasts indicates that whole serum or lipid deficient serum, plus fatty acids, reduces the level of acetyl-CoA carboxylase. In their experiments, linoleic acid was much less effective than palmitic acid in reducing the amount of radioactive acetate incorporated into total lipids.

One of the more important aspects of these data is the demonstrated response of monoenoic acid biosynthesis to dietary lipids in these cells. Previous studies have shown that lipogenesis, as determined by ^{14}C-acetate incorporation into fatty acids, in mouse and rat hepatoma was not affected by dietary fat (22,26-28). The apparent discrepancy between the mass data from these cultured hepatoma cells and the radioisotope data from host grown hepatomas is not understood but is the subject of further investigations.

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REFERENCES

1. Wood, R., Lipids 8:690 (1973).
2. Rouser, G., A.M. Siakotos and S. Fleischer, Ibid. 1:85 (1966).
3. Dittmer, J.C., and R.L. Lester, J. Lipid Res. 5:126 (1964).
4. Wood, R., Lipids 7:596 (1972).
5. Rouser, G., G.J. Nelson, S. Fleischer and G. Simon, in "Biological Membranes," Edited by D. Chapman, Academic Press, New York, 1968, p. 5.
6. Wood, R., and R.D. Harlow, Arch. Biochem. Biophys. 135:272 (1969).
7. Bergelson, L.D., E.V. Dyatlovitskaya, T.I. Torkhovskaya, I.B. Sorokina and N.P. Gorkova, Biochim. Biophys. Acta. 210:287 (1970).
8. Selkirk, J.K., J.C. Elwood and H.P. Morris, Cancer Res. 31:27 (1971).
9. Wood, R., and R.D. Harlow, Arch. Biochem. Biophys. 131:494 (1969).
10. Kuksis, A., L. Marai, W.C. Breckenridge, D.A. Gornall and O. Stachnyk, Can. J. Physiol. Pharmacol. 46:511 (1968).
11. Getz, G.S., W. Bartley, F. Stirpe, B.M. Nolton and A. Renshaw, Biochem. J. 80:176 (1961).
12. Dittmer, J.C., and D.J. Hanahan, J. Biol. Chem. 234:1976 (1959).
13. Ruggieri, S., and A. Fallani, in "Tumor Lipids: Biochemistry and Metabolism," Edited by R. Wood, American Oil Chemists' Society, Champaign, Ill., 1973, p. 89.

14. Bergelson, L.D., and E.V. Dyatlovitskaya, Ibid., p. 111.
15. van Hoeven, R.P., and P. Emmelot, Ibid., p. 126.
16. Ruggieri, S., and A. Fallani, Lo Sperimentale 118:483 (1968).
17. Wood, R., and R.D. Harlow, Arch. Biochem. Biophys. 135:272 (1969).
18. Wood, R., and R.D. Harlow, Ibid. 141:183 (1970).
19. Bailey, J.M., B.V. Howard, L.M. Dunbar and S.F. Tillman, Lipids 7:125 (1972).
20. Wiegand, R.D., and R. Wood, In press.
21. Allmann, D.W., and D.M. Gibson, J. Lipid Res. 6:51 (1965).
22. Abraham, S., Amer. J. Clin. Nutr. 23:1120 (1970).
23. Bartley, J.C., and S. Abraham, Biochim. Biophys. Acta 280:258 (1972).
24. Rothblat, G.H., Advan. Lipid Res. 7:135 (1969).
25. Jacobs, R.A., W.S. Sly and P.W. Majerus, J. Biol. Chem. 248:1268 (1973).
26. Sabine, J.R., S. Abraham and H.P. Morris, Cancer Res. 28:46 (1968).
27. Sabine, J.R., S. Abraham and I.L. Chaikoff, Biochim. Biophys. Acta 116:407 (1966).
28. Sabine, J.R., S. Abraham and I.L. Chaikoff, Cancer Res. 27:793 (1967).

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Regulatory Function of Mitochondria in Lipogenesis[1]

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ABSTRACT

A cell-free system, consisting of particle-free rat liver cytoplasm plus purified mitochondria, was used to study the synthesis of fatty acid from ^{14}C-U-alanine. Incorporation of label into lipid was dependent upon the presence of both cytoplasm and mitochondria. However, the metabolic state of the animal, prior to sacrifice, determined the capacity of various mitochondrial preparations to reconstitute the system. Mitochondria prepared from the livers of starved, fat-fed, or alloxan-diabetic rats failed to support fatty acid synthesis to the same extent as mitochondria prepared from control animals. Likewise, less $^{14}CO_2$ was produced in the system reconstituted with liver mitochondria prepared from starved rats. Possible mechanisms are discussed.

INTRODUCTION

The enzymes of both the cytoplasmic and mitochondrial compartments of the liver cell are required for the synthesis of fatty acid from glucose (see [1] for a review of pertinent literature). The glycolytic enzymes of the cytoplasm catalyze the production of pyruvate, which the mitochondrial enzymes convert into oxaloacetate and acetyl CoA, which then condense to form citrate. This tricarboxylic acid escapes the mitochondrion to be cleaved in the cytoplasm to oxaloacetate plus acetyl CoA. The latter is utilized by the cytoplasmic enzymes as an "activated" two carbon fragment for fatty acid synthesis.

The mitochondrion carries out at least three key steps during the process of lipogenesis: the oxidative decarboxylation of pyruvate to acetyl CoA by the pyruvate dehydrogenase complex, the condensation of acetyl CoA with oxaloacetate to give citrate by citrate synthase, and the exchange of citrate for extramitochondrial malate by an exchange-diffusion carrier located within the mitochondrial inner membrane. The simplest system for studying the participation of the mitochondrion in the process of lipogenesis would be a cell-free system consisting of particle-free cytoplasm and mitochondria prepared from rat liver. Such systems have been described (2,3) but were used mainly to study the incorporation of labeled acetate into fatty acids. Watson and Lowenstein (4) have described a new system in which the incorporation of label from alanine (source of pyruvate by transamination) into fatty acids was studied. Particle-free cytoplasm prepared from livers of rats induced into a high lipogenic state was used. Reconstitution of fatty acid synthesis by the system was demonstrated to be dependent upon the presence of mitochondria. This system allows a more complete investigation of the role of the mitochondrion because incorporation of label into fatty acid demands a flow of carbon through enzymatic steps within the matrix space. The Watson-Lowenstein cell-free system has been used in the present study to demonstrate that the capacity of hepatic mitochondria to support lipogenesis is affected by the metabolic state of the animal prior to sacrifice.

METHODS AND MATERIALS

Preparation of Mitochondria and "Superlipogenic Supernatant"

Mitochondria were prepared from rat liver by the method of Johnson and Lardy (5). A 105,000 x g supernatant was prepared from the livers of rats which had been 48 hr starved and then 48-72 hr refed ad libitum on a 68% sucrose, fat-free diet obtained from Nutritional Biochemicals (6). The 105,000 x g supernatant (hereafter called "superlipogenic supernatant") was activated as described by Watson and Lowenstein (4). This preparation of soluble enzymes was found stable to freezing in a liquid nitrogen storage tank for at least 6 weeks. Protein was determined by the biuret method (7) and on occasion by the Lowry method (8).

Reconstituted Cell-Free System Capable of Lipogenesis

The incubation conditions used in the recon-

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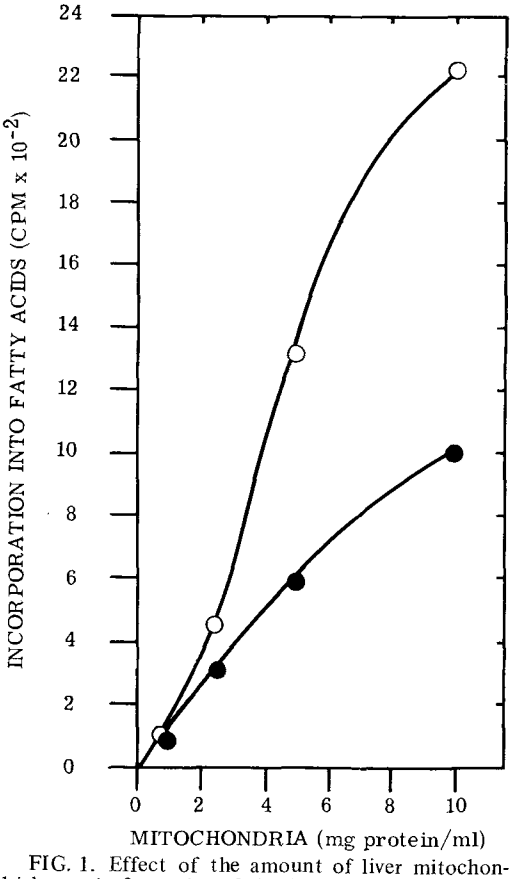

FIG. 1. Effect of the amount of liver mitochondrial protein from chow-fed (○) and 48 hr starved (●) rats used to reconstitute the Watson-Lowenstein soluble system for fatty acid synthesis.

stituted system have been described by Watson and Lowenstein (4). A major difference was that liver mitochondria were prepared from rats in various defined metabolic states and added to the "superlipogenic supernatant" in the reconstituted system. The incubations were carried out for 30 min in 25 ml Erlenmeyer flasks (stoppered with serum caps) at 37 C with air as the gas phase and constant shaking (120 cycle/min). The incubation mixtures (1.5 ml) contained 20 mM $KHCO_3$, 9 mM dithiothreitol, 12 mM $MgCl_2$, 4 mM L-malate, 2 mM adenosine triphosphate (ATP), 4 mM glucose-6-phosphate, 5 mM α-ketoglutarate, 4 mM phosphate buffer (pH 7.4), 12.5 mM glycylglycine-NaOH buffer (pH 7.4), ca. 50 mM sucrose, 6 mM ^{14}C-U-alanine (100-200 cpm/nmole), 0.662 mg nicotinamide adenine dinucleotide phosphate (NADP), .0945 mg nicotinamide adenine dinucleotide (NAD), 0.2 mg Coenzyme A, ca. 4.5 mg of activated "superlipogenic supernatant" protein, and 7.5 mg of mitochondrial protein (unless otherwise indicated). When tritiated water incorporation into fatty acids was studied, labeled alanine was omitted, and each flask received ca. 3.75 mc of ^{3}H-water.

Fatty acids were extracted from the incubation medium after saponification, as described by Watson and Lowenstein (4). The extracts were evaporated to dryness in scintillation vials and counted in a toluene base scintillation fluid (0.01% p-bis[2-(5-phenyloxazolyl)]-benzene and 0.4% 2,5-diphenyloxazole) with a Packard Tri-Carb scintillation counter. Radioactive CO_2 was collected in hyamine hydroxide after acidification of the incubation medium (center wells from Kontes Glass Company, Vineland, N.J.) and counted in the scintillation fluid described above. Results were analyzed for statistical significance by a two-tailed student's *t* test.

Assay of Respiratory Control and State 3 Respiration

State 3 respiration and the respiratory control ratio always were assayed as a check on the integrity of the mitochondrial preparations (9). The incubation mixture used (5 ml) contained 1125 μmoles of sucrose, 50 μmoles of Pi (pH 7.4), 25 μmoles of $MgCl_2$, 100 μmoles of KCl, 100 μmoles of triethanolamine (pH 7.4), 25 μmoles of glutamate, 25 μmoles of L-malate, and 10-12 mg of mitochondrial protein. Respiration was released with 1 μmole of adenosine diphosphate (ADP).

Diet and Rats

Male rats of the Wistar strain (185-250 g) were used in all experiments. The animals were maintained on stock colony diet (chow-fed), unless otherwise indicated. The high carbohydrate, fat-free diet was 68% in sucrose, 21% in casein, 5% in alphacel cellulose, and supplemented with salts and vitamins. The high fat, carbohydrate-free diet was 58% in corn oil margarine, 25% in casein, 11% in alphacel cellulose, and also supplemented with salts and vitamins.

RESULTS

The incorporation of labeled alanine into fatty acids by the cell-free system was roughly proportional to the mitochondrial protein concentration of the incubation medium (Fig. 1). Typical time course studies are shown in Figure 2 for the synthesis of labeled fatty acids and carbon dioxide. A lag was observed in the rate of fatty acid formation which may reflect the time required for generation of a critical concentration of citrate for fatty acid synthesis (4). In the studies shown in Figures 1 and 2, less incorporation of labeled alanine into fatty acids

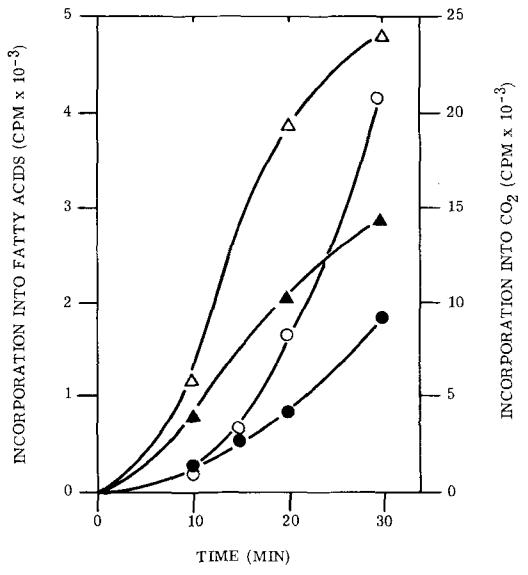

FIG. 2. Time course study for fatty acid synthesis (○) and $^{14}CO_2$ production (△) in the reconstituted system for liver mitochondria prepared from chow-fed (○,△) and 48 hr starved (●,▲) rats.

and carbon dioxide was noted when mitochondria prepared from the liver of 48 hr starved rats were used to reconstitute fatty acid synthesis. Since statistical evaluation of this difference was highly desirable, techniques were developed for the preparation and assay of up to 12 mitochondrial preparations in a day.

As shown in Table I, liver mitochondria prepared from chow-fed animals had significantly greater capacity to support fatty acid synthesis than mitochondria prepared from 48 hr starved animals. Likewise, less conversion of labeled alanine into carbon dioxide was observed.

The incorporation of tritiated water into fatty acids was also significantly less when mitochondria from starved animals were used to reconstitute fatty acid synthesis. In an experiment involving three preparations of liver mitochondria from chow-fed rats and three preparations from 48 hr starved rats, much less tritiated water was incorporated into fatty acids when the system was reconstituted with mitochondria from starved rats (1870 ± 330 cpm/flask for chow-fed vs. 840 ± 140 cpm/flask for starved, $P < 0.05$). In another experiment using single preparations of mitochondria from chow-fed and 48 hr starved animals to reconstitute fatty acid synthesis, the latter preparation supported 87% less ^{14}C-U-alanine incorporation into fatty acids, 65% less ^{14}C-U-alanine conversion to $^{14}CO_2$, and 74% less tritiated water incorporation into fatty acids.

TABLE I

Reconstitution of Fatty Acid Synthesis with Liver Mitochondria Prepared from Chow-Fed and Starved Rats

Mitochondrial source	^{14}C-U-Alanine to:	
	CO_2	Fatty acids
	nmoles/min/mg protein	
Chow-fed (4)	8.3 ± 0.96[a]	0.98 ± 0.13
Starved[b] (4)	3.2 ± 0.18	0.22 ± 0.03
P	< .01	< .01

[a]Standard error of the mean.

[b]Rats were starved for 48 hr prior to sacrifice.

Refeeding 48 hr starved rats with a high fat diet for 48 hr failed to restore the original capacity of the mitochondria to reconstitute fatty acid synthesis (Table II). However, refeeding starved rats with a high carbohydrate diet for 48 hr largely restored the capacity of the mitochondria to support fatty acid synthesis in the reconstituted system. The rate of ^{14}C-U-alanine oxidation to $^{14}CO_2$ was also greater with liver mitochondrial preparations from animals refed carbohydrate rather than fat (Table II).

Mitochondria prepared from the livers of rats maintained on a high fat diet for six days were also defective in reconstituting fatty acid synthesis (Table III), as were mitochondria prepared from alloxan-diabetic rats (Table IV). Again, a decreased capacity to oxidize ^{14}C-U-alanine to carbon dioxide was noted with these mitochondrial preparations.

DISCUSSION

This study was designed to compare the capacity of liver mitochondria prepared from

TABLE II

Reconstitution of Fatty Acid Synthesis with Liver Mitochondria Prepared from Chow-Fed, Fat-Refed and Carbohydrate-Refed Rats

Mitochondria source	^{14}C-U-Alanine to:	
	CO_2	Fatty acids
	nmoles/min/mg protein	
Chow-fed (4)	8.3 ± 0.96[a]	0.98 ± 0.13
Refed fat (4)[b]	2.4 ± 1.1[c]	0.23 ± 0.10[c]
Refed carbohydrate (3)[b]	5.2 ± 0.76	0.76 ± 0.05

[a]Standard error of the mean.

[b]Rats which had been starved for 48 hr were refed for 48 hr on either the high fat diet or the high carbohydrate diet described in the text.

[c]$p < .01$ from chow-fed mitochondria.

TABLE III

Reconstitution of Fatty Acid Synthesis with Liver Mitochondria Prepared from Chow-Fed and Fat-Fed Rats

Mitochondrial source	^{14}C-U-Alanine to:	
	CO_2	Fatty acids
	nmoles/min/mg protein	
Chow-fed (4)	7.9 ± 0.86[a]	0.79 ± 0.06
Fat-fed[b] (4)	2.0 ± 0.25	0.19 ± 0.03
P	< .001	< .001

[a]Standard error of the mean.

[b]Rats were maintained on a high fat diet for six days prior to sacrifice.

TABLE IV

Reconstitution of Fatty Acid Synthesis with Liver Mitochondria Prepared from Chow-Fed and Alloxan-Diabetic Rats

Mitochondrial source	^{14}C-U-Alanine to:	
	CO_2	Fatty acids
	nmoles/min/mg protein	
Chow-fed (6)	6.9 ± 1.1[a]	0.71 ± 0.08
Diabetic[b] (5)	3.0 ± 0.3	0.36 ± 0.04
P	< 0.02	< 0.02

[a]Standard error of the mean.

[b]Diabetic rats were produced by the iv injection of alloxan (40 mg/kg). Rats were maintained for one to four weeks on adequate insulin to maintain normal blood glucose. Two days before sacrifice, insulin was withdrawn. Animals with blood glucose values greater than 300 mg % were used in this study.

animals in various metabolic states to reconstitute fatty acid synthesis in a soluble system. It was found that mitochondria prepared from liver tissue of animals which were both gluconeogenic and ketogenic failed to support fatty acid synthesis to the same extent as mitochondria from chow-fed or high carbohydrate fed animals. Thus, mitochondria prepared from starved, fat-fed, starved and refed fat, or alloxan-diabetic animals appeared defective in capacity to reconstitute the Watson-Lowenstein soluble system. In other words, mitochondria prepared from liver, which was actively engaged in the process of de novo lipogenesis, reconstituted fatty acid synthesis to a much greater extent than mitochondria prepared from liver more actively engaged in the production of glucose and ketone bodies. These results lead us to propose that the mitochondrion undergoes metabolic adaptations which influence the capacity of these organelles to support the process of fatty acid synthesis.

Throughout the course of this study, much caution was taken to insure that the difference noted between normal and ketotic animals was not merely apparent but real. For example, an error in the estimation of the protein concentration of the mitochondrial preparation might explain the results. However, estimation of the protein concentration by the Lowry method (8) gave the same results as reported here using the biuret method. Likewise, correction of the biuret method for any turbidity by the potassium cyanide method of Keyser and Vaughn (10) failed to affect the results obtained. It also seemed conceivable that the mitochondrial preparations from ketotic animals might be contaminated to a greater extent with endoplasmic reticulum. However, when mitochondria and microsomes were prepared from liver of normal and starved animals, both mitochondrial preparations were consistently found to be contaminated to the extent of ca. 5%. Calculations were made on the basis of the microsomal marker enzyme glucose-6-phosphatase using the specific activities of microsomal preparations and the total activities of mitochondrial preparations.

The studies of Watson and Lowenstein (4) suggested that de novo fatty acid synthesis, rather than chain elongation, was measured by the reconstituted system. This conclusion was confirmed in the present study. Particle-free cytoplasm prepared from liver low in fatty acid synthetase and acetyl CoA carboxylase, e.g. starved animals, failed to support fatty acid synthesis. Thus, the Watson-Lowenstein reconstituted system can be used to investigate the regulatory function of the mitochondrion in de novo fatty acid synthesis.

There also has been much concern during the course of this study as to whether the differences noted between mitochondrial preparations were induced during isolation of these organelles from the tissue. Preparation of intact mitochondria from any tissue is recognized as more of an art than a science, making isolation artifacts very difficult to rule out. The mitochondria prepared for this study were characterized by high respiratory control ratios (from 4-7), nearly theoretical ADP/O ratios (greater than 2.5), and low intrinsic ATPase activities. However, consistently lower respiratory control ratios, state 3 rates of respiration, and ADP/O ratios were observed with mitochondria prepared from ketotic animals. These results will be the subject of another communication which will characterize these mitochondria in greater detail with respect to biochemical properties. In the present experiments, all mitochondria were prepared as quickly as possible and assayed immediately for the capacity to reconstitute

fatty acid synthesis. No evidence was found for any difference in the rate of aging of mitochondria prepared from livers of normal or starved rats. Other isolation procedures for liver mitochondria currently are being explored to determine whether the results obtained are a function of the technique used to disrupt the liver. Although an exhaustive investigation has not been conducted yet, other isolation methods and conditions which yield relatively intact mitochondria appear to give results similar to those reported here. Therefore, no evidence has been found which would suggest that the difference noted between mitochondrial preparations from normal and ketotic animals was induced during isolation of the mitochondria. However, definitive evidence against this possibility has not been obtained.

The basis for the decreased capacity of mitochondria prepared from ketotic animals to reconstitute fatty acid synthesis remains to be established. The possibility that the results are due to greater dilution of the specific activity of the labeled precursors has been eliminated. The incorporation of tritiated water into fatty acids was found to be less with the mitochondria shown to be defective in reconstituting fatty acid synthesis from ^{14}C-U-alanine. Since incorporation of water is not subject to dilution effects, less carbon clearly is incorporated into fatty acids in the presence of mitochondria prepared from the liver of starved, fat-fed, and diabetic animals.

The interconvertible enzyme pyruvate dehydrogenase of the mitochondrial matrix space may be involved in the difference reported here among mitochondrial preparations. This enzyme has been shown by Linn et al. (11) and Wieland et al. (12) to undergo interconversion between an active, dephosphorylated form of the enzyme and an inactive, phosphorylated form. Activation is catalyzed by a phosphatase; inactivation, by a protein kinase. Wieland et al. (13) have demonstrated that liver pyruvate dehydrogenase is largely in the inactive state in ketotic animals and has greater activity in normal animals. Thus, the results of the present study may be explained in part by the interconvertibility of the pyruvate dehydrogenase complex. Mitochondria prepared from starved, fat-fed, or diabetic animals could be expected to have a lower amount of active pyruvate dehydrogenase. Indeed, this would account for the decrease in $^{14}CO_2$ formation observed when mitochondria from these animals were used to reconstitute lipogenesis. Studies currently are being conducted in this laboratory on the pyruvate dehydrogenase activity of isolated mitochondria from normal and starved animals (14). Initial studies have not been definitive because of difficulties encountered with the assay of this enzyme complex in liver mitochondria.

There are a number of other possible explanations which are under investigation. Citrate is the immediate precursor of cytoplasmic acetyl CoA needed for fatty acid synthesis (4). Citrate crosses the mitochondrial inner membrane on a citrate-malate antiport. Any defect in this transport system would be expected to curtail lipogenesis. Another possible mechanism involves the relationship between lipogenesis and the energy state of the cell. The mitochondria would appear to regulate the cytoplasmic phosphate potential (ATP/ADP x Pi) which in turn, via equilibrium enzymes, influences and is influenced by the NAD^+ and $NADP^+$ redox states (15). Therefore, it appears conceivable that a difference in the cytoplasmic phosphate potential maintained by a given mitochondrial preparation might have a profound influence upon metabolic processes, such as fatty acid synthesis. One reason this should be considered as a possibile regulatory feature is that mitochondria prepared from ketotic animals have been found to have lower respiratory control and ADP/O ratios (14). The stimulatory effect of mitochondria on acetate incorporation into fatty acids in a cell-free system reported by Bhaduri and Srere (2) and Iliffe and Myant (3) also may be relevant. The acetyl CoA carboxylase step was made rate-limiting by the rate of ATP production by the mitochondrion (3). The capacity of various mitochondrial preparations to maintain the phosphate potential of the reconstituted system currently is being investigated in this laboratory.

Would such an apparent defect in mitochondrial function, as demonstrated in the present study, be physiologically important? It is believed that the rate limiting step of fatty acid synthesis is catalyzed by acetyl CoA carboxylase. However, there are very important metabolic adaptations which occur among the other enzymes involved in fatty acid synthesis (see [16] for a review of pertinent literature). It is proposed here that although the changes may be less marked than those that occur in the cytosol, the liver mitochondrion also may undergo metabolic adaptation with respect to its capacity to support lipogenesis.

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REFERENCES

1. "Metabolic Roles of Citrate," Edited by T.W.

Goodwin, Academic Press, New York, 1968, p. 41, 61, 123.
2. Bhaduri, A., and P.A. Srere, J. Biol. Chem. 239:1357 (1964).
3. Iliffe, J., and N.B. Myant, Biochem. J. 91:369 (1964).
4. Watson, J.A., and J.M. Lowenstein, J. Biol. Chem. 245:5993 (1970).
5. Johnson, D., and H. Lardy, in "Methods of Enzymology," Vol. X, Edited by R.W. Estabrook and M.E. Pullman, Academic Press, New York, 1967, p. 317.
6. Wolley, J.G., and W.H. Sebrell, J. Nutrition 39:191 (1945).
7. Gornall, A.G., C.H. Bardawill and M.M. David, J. Biol. Chem. 177:751 (1949).
8. Lowry, O.H., W.J. Rosebrough, A.L. Farr and R.J. Randall, Ibid. 193:265 (1951).
9. Estabrook, R.W., in "Methods in Enzymology," Vol. X, Edited by R.W. Estabrook and M.E. Pullman, Academic Press, New York, 1967, p. 41.
10. Keyser, J.W., and J. Vaughn, Biochem. J. 44:XXII (1949).
11. Linn, T.C., F.H. Pettit, F. Hucho and L.J. Reed, Proc. Nat. Acad. Sci., U.S.A. 64:227 (1969).
12. Wieland, O., and E. Siess, Proc. Nat. Acad. Sci., U.S.A. 65:947 (1970).
13. Wieland, O., E. Siess, F.H. Schulze-Wethmar, H.G.V. Funche and B. Winto, Arch. Biochem. Biophys. 143:593 (1971).
14. Harris, R.A., J.M. Mahoney, J.P. Mapes and M.C. Leland, in "Studies on the Regulatory Role of the Mitochondrion in Lipogenesis," Federation of American Societies for Experimental Biology, Atlantic City, Abstr. 2584, April 1973.
15. Veech, R.C., L. Raijman and H.A. Krebs, Biochem. J. 117:499 (1970).
16. Gibson, D.M., R.T. Lyons, D.F. Scott and Y. Muto, in "Advances in Enzyme Regulation," Vol. 10, Edited by G. Weber, Pergamon Press, Oxford, 1972, p. 187.

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Stimulation of Ion Transport by Phosphoglycerides[1]

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ABSTRACT

Phosphoglyceride extracts of beef heart mitochondria stimulated the transport of cations and "permeable" anions across a membrane barrier of chloroform in a U-tube. Neutral lipids fractionated from total lipid extracts of heart mitochondria were essentially void of transport activity. Remarkable differences were noted between the capacity of the various phospholipids to support transport of ions in the U-tube system.

INTRODUCTION

The importance of phosphoglycerides with respect to membrane phenomena has been of interest for over four decades (1,2). The structure and function of biological membranes are determined in large part by phosphoglycerides (3-5). Because of the inherent capacity to form bilayers with permeability characteristics similar to natural membranes, these lipids have long been considered a determinant of membrane permeability. The nonpolar tails of the phosphoglycerides associate within bilayers and biological membranes to form a "hydrophobic core," with limited permeability to cations and anions. Also, because they form lipid-soluble undissociated salts with various cations, phosphoglycerides repeatedly have been suggested as participating possibly in cation transport (6,7).

The present study was prompted primarily by the report by Blondin et al. (8) of the isolation of a neutral ionophore from beef heart mitochondria. Initial efforts in this laboratory to purify this ionophore have not been successful; however, a number of phosphoglycerides have been found to transport ions across a nonpolar membrane phase used in our laboratory to assay for ionophores. Since both neutral and anionic ionophores are also active in the assay system used, the observations are relevant to the role phosphoglycerides may play as ion carriers.

METHODS AND MATERIALS

Lipids

Total lipid was extracted from isolated beef heart mitochondria essentially as described by Rouser and Fleischer (9). Neutral lipids were separated from phosphoglycerides by silicic acid chromatography as described previously (10). Individual phosphoglycerides were purified by chromatography on silicic acid and diethylaminoethyl (DEAE) cellulose as described by Rouser et al. (11). Purity of the fractions was established by thin layer chromatography (TLC) (12) with Silica Gel H plates prepared in 1 mM Na_2CO_3 and developed in $CHCl_3$-MeOH-HOAC-H_2O (80:13:8:0.3). Spots were detected by charring after exposure of the plates to a 0.6% solution of sodium dichromate in 55% (by wt) sulfuric acid. Lipids were applied to the plates in quantities which allowed the detection of 1% impurity. The cardiolipin and choline phosphoglyceride preparations used in this study were chromatographically homogeneous. Ethanolamine phosphoglyceride was 5% contaminated with other phospholipids (probably cardiolipin and serine phosphoglyceride). In some studies, chromatographically homogeneous cardiolipin prepared from beef hearts by Sylvana Chemical Co., Orange, N.J. was used. Sphingomyelin was a product of General Biochemicals, Chagrin Falls, Ohio. Inositol phosphoglyceride was a chromatographically homogeneous product from plants (Applied Science Laboratories, State College, Pa.). A sample of nigericin (free acid) was provided by D. Wong (Eli Lilly Co., Indianapolis, Ind.). Valinomycin was purchased from Calbiochem (California). Ketamine hydrochloride ("Ketalor"; 2-[0-chlorophenyl]-2-[methylamino] cyclohexanone hydrochloride) was obtained from Park-Davis Laboratories.

Experimental System

The U-tube system developed by Pinkerton et al. (13) was adapted for these studies. The apparatus consisted of a U-tube (Ace Glass, 150 mm) which had two 5 ml aqueous layers separated by 20 ml of chloroform. A typical experiment consisted of placing chloroform in the U-tube along with the test substance, e.g. a neutral ionophore or various lipid fractions. Then a solution 0.15 M in KCl and 0.1 mM in potassium picrate, pH 7.0, was placed in the left arm of the U-tube; distilled deionized water was placed in the right arm. A Dubnoff shaking water bath operated at room temperature at 80 cycles/min was used to create a rocking motion of the contents of the tubes. The concentration of picrate in the aqueous compartments was

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TABLE I

Stimulation of K^+ and Picrate Transport by Valinomycin and Nigericin

Test substance[a]	Transported species	
	Picrate	Potassium
	nmoles in 14 days	
None	51	0
Valinomycin	458	1005
Nigericin	347	975

[a]U-tubes contained two aqueous compartments (5 ml) separated by 20 ml chloroform. The left solution was 0.15 M in KCl and 0.1 mM in potassium picrate at pH 7.0. The right side contained 5 ml deionized distilled water. Additions: 0.2 μmoles of valinomycin; 0.5 μmoles of nigericin (free acid). Results are expressed as the average of duplicate U-tubes, Expt. 62871.

determined by the absorbance at 356 nm and the molar extinction coefficient of 13,700 (14). Sodium and potassium were determined with an atomic absorption spectrophotometer (Instrumentation Laboratory Model No. 153).

RESULTS

Stimulation of Picrate Transport by Ionophores

Pinkerton et al. (13) demonstrated that valinomycin would bring about the transport of picrate against a concentration gradient in a U-tube with a chloroform barrier. The results given in Table I are consistent with these observations and demonstrate that the anionic ionophore nigericin also will support picrate transport. Since 500 nmoles picrate were placed in 5 ml of 0.15 M KCl in the left arm of the U-tube, picrate was transported against a concentration gradient after 250 nmoles of picrate appeared in the 5 ml of distilled water present in the right arm of the U-tube. Although both ionophores stimulate K^+ transport, there is a basic difference in the transport mechanisms since nigericin carries a negative charge (14-17), whereas valinomycin is a neutral ionophore (see Discussion).

Picrate Transport by Phosphoglycerides

Total lipids extracted with chloroform-methanol from beef heart mitochondria also were found to stimulate picrate and K^+ translocation in the U-tube system (Table II). Fractionation of the total lipid extract into neutral lipid and phosphoglyceride fractions by silicic acid chromatography demonstrated that the transport mediating activity resided almost entirely with the phosphoglyceride fraction. Fractionation of the mitochondrial phosphoglycerides into major components revealed that cardiolipin, ethanolamine phosphoglyceride, inositol phosphoglyceride, and choline phosphoglyceride were active to varying degrees for picrate transport (Table II). The minor components tested (phosphatidic acid and sphingomyelin) also exhibited activity. Under the conditions of these experiments, cardiolipin, inositol phosphoglyceride, ethanolamine phosphoglyceride, and phosphatidic acid were much more active than choline phosphoglyceride or sphingomyelin. This relationship was also apparent in a time study (Fig. 1). Transport of picrate against a concentration gradient was observed with cardiolipin and ethanolamine phosphoglyceride but could not be demonstrated with choline phosphoglyceride. Lipid peroxidation was not a factor because experiments conducted under a flowing nitrogen atmosphere gave results similar to those reported in Table II.

TABLE II

Stimulation of K^+ and Picrate Transport by Total Lipid Extracts, Phosphoglyceride Extracts, and Purified Phospholipids

Test substance[a]	Transported species	
	Picrate	Potassium
	nmoles in 14 days	
None	51	0
HBHM total lipids	361	835
HBHM neutral lipids	54	0
HBHM phospholipids	345	640
Cardiolipin	465	7830
Ethanolamine phosphoglyceride	342	1415
Inositol phosphoglyceride	405	1665
Phosphatidic acid	319	2310
Choline phosphoglyceride	112	273
Sphingomyelin	25	70

[a]U-tube system described in Table I was used. Ten mg of each lipid or lipid fraction was added to the chloroform layer. Results are expressed as the average of duplicate tubes except for the U-tubes containing inositol phosphoglyceride and phosphatidic acid where the amounts needed were not available. Expts. 62871 and 71671.

Table III demonstrates that little picrate accumulated in the chloroform barrier when valinomycin and cardiolipin were used to stimulate transport. On the other hand, large amounts of picrate appeared in the chloroform with choline phosphoglyceride as the test carrier. High concentrations of ketamine, a secondary amine with general anesthetic properties, inhibited picrate transport in the U-tube system by carrying essentially all of the picrate into the chloroform phase. Ethanolamine phosphoglyceride bound a significant amount of the picrate in the chloroform phase but much less than choline phosphoglyceride.

Stimulation of Countertransport by Cardiolipin

Cardiolipin was shown to stimulate counter

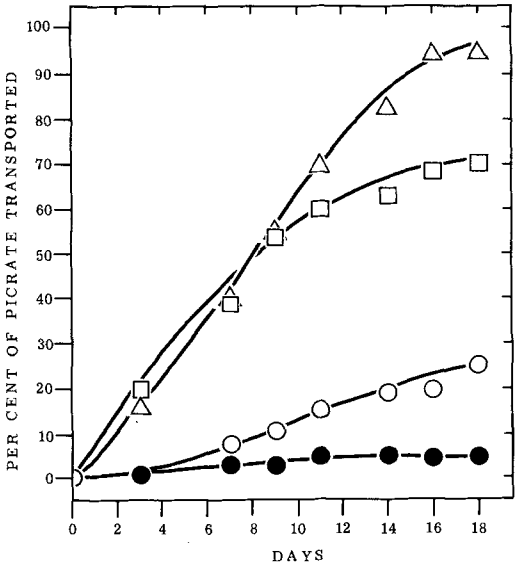

FIG. 1. Time course of picrate transport by purified phosphoglycerides. U-tubes contained two aqueous compartments separated by 20 ml chloroform. The left solution was 0.15 M in KCl and 0.1 mM in potassium picrate at an initial pH of 7.0. Right side contained 5 ml deionized, distilled water. Chloroform layer contained: (△, cardiolipin (24.4 mg); (□), ethanolamine phosphoglyceride (25 mg); (○), choline phosphoglyceride (26 mg); or (●), no additions. Results are expressed on the basis of the per cent of the total picrate on the left side that was transported to the right aqueous compartment. Expt. 2271H.

exchange of Na^+ and K^+ across a chloroform barrier in the experiment summarized in Table IV. This exchange activity also could be demonstrated with the other phosphoglycerides which were active in the transport system containing picrate.

DISCUSSION

Stimulation of Transport Across a Bulk Phase by Valinomycin and Nigericin

As established by the work of others (13-18), valinomycin supports transport in the U-tube system used in this study by the following mechanism. The neutral ionophore molecule combines with K^+ to form a charged binary complex which in turn combines with the proper anion to form a neutral molecule soluble in the chloroform membrane phase. Chloride and acetate are not suitable anions but picrate and a number of other lipophilic anions of strong acids (e.g. 8-anilino-1-naphthalene sulfonate) will function to neutralize the charge of the valinomycin-K^+ complex. Since K^+ and picrate are transported together without the transport of chloride, a concentration gradient of KCl will drive the accumulation of picrate against its concentration gradient (13,15).

TABLE III

Accumulation of Picrate into the Chloroform Barrier of the U-tube in the Presence of Valinomycin and Various Phospholipids

Test substance[a]	Picrate transported into: $CHCl_3$ layer	Picrate transported into: H_2O layer
	nmoles in 9 days	
None	< 5	20
Valinomycin	19	398
Cardiolipin	< 5	322
Ethanolamine phosphoglyceride	86	208
Choline phosphoglyceride	346	65
Ketamine	453	28

[a]Experimental design is described in the legend of Figure 1. Other additions were: 0.5 μmoles of valinomycin; and 2.5 mg of ketamine (to the right hand side only). Chloroform values were obtained from the difference between the total picrate minus that accounted for in the left and right hand sides. Expts. 20971 and 22771.

The mechanism of stimulation of transport by anionic ionophores, such as nigericin, stands in contrast to that of neutral ionophores (13-18). The anionic ionophores combine with either a cation or a proton to form a neutral complex soluble in the chloroform membrane phase. Although nigericin does not combine with picrate, it brought about the accumulation of picrate against a concentration gradient under the conditions of this study. Two mechanisms can be proposed as possible explanations for these results: (a) countertransport of protons for potassium by nigericin will result in the establishment of a pH gradient. As the pH on the left side decreases, picric acid forms. The latter would be soluble in chloroform and

TABLE IV

Stimulation of Countertransport of Na^+ and K^+ by Cardiolipin

Test substance[a]	Transported ion[b] Na^+	Transported ion[b] K^+
	μmoles in 14 days	
None	3.55	0.45
Cardiolipin	48.7	59.4

[a]The left solution (5 ml) of the U-tube was 150 mM in KCl. The right solution (5 ml) was 150 mM in NaCl. Cardiolipin (11.4 μmoles) was added to the chloroform layer (20 ml).

[b]Refers to either the amount of Na^+ appearing in the side of the U-tube originally charged with KCl or the amount of K^+ appearing in the side of the U-tube originally charged with NaCl. Expt. 62971F.

would migrate across the barrier to react with the hydroxyl ion on the other side of the chloroform phase to yield picrate; (b) transport of potassium across chloroform by nigericin down a substantial concentration gradient would be expected to produce a potential across the chloroform layer, positive on the right side and negative on the left. The result would be a driving force for the movement of picrate across the chloroform barrier. In our opinion, further studies are required to differentiate between these two mechanisms.

Possible Transport Mechanisms of Phosphoglycerides

Phosphoglycerides have been shown in this study to support the transport of K^+, Na^+, and picrate across a chloroform barrier. The evidence suggests that the mechanism may be similar to that of nigericin, i.e. cations are carried by the phospholipid, whereas anions are either transported as the neutral species on a pH gradient or driven by a potential produced by the movement of cations down a large concentration gradient. Support for this comes from the following observations. (a) Only those phosphoglycerides which would have the same charge (negative) as nigericin at physiological pH were active as carriers. (The inactive lipids tested included choline phosphoglyceride and sphingomyelin, which are zwitter ions, and a number of neutral lipids, which are not charged. Acidic impurities [see Methods] may have made the ethanolamine phosphoglyceride behave as an acidic lipid.) (b) The phosphoglycerides catalyzed Na^+ and K^+ counterexchange across a chloroform barrier, as would be expected if there was not a high degree of specificity for the nature of the cation. Indeed, the studies of Schulman et al. (19,20) a number of years ago clearly demonstrated that phosphoglycerides would stimulate countertransport of Na^+ and K^+ across an organic solvent barrier. In the present study, no evidence was found for the presence of neutral or cationic ionophores in lipid extracts of beef heart. A cationic ionophore would not be expected to support picrate transport against a concentration gradient, as was observed under the conditions used in this study. The possibility that the phosphoglycerides were contaminated by a neutral ionophore is difficult to rule out, but this seems unlikely.

An alternative mechanism would be that electrolytes might be transported by the lipid in the form of aqueous pockets within polymeric lipid structures. Phase inversion of the lipid at the interface surfaces would allow uptake and release of the salts in the aqueous compartments. By this mechanism, the tendency of the lipid for phase inversion would be a critical determinant of the capacity of the various lipids to support transport. Lipids which did not invert readily at the interfaces would not be expected to support transport by this mechanism.

Thus, it is obvious that additional studies must be carried out to establish the basis for the transport of ions by phosphoglycerides demonstrated in the present study. It would be of great interest to determine whether chloride is transported under the conditions used and to establish the stoichiometry between total cation, proton, anion, and hydroxide transport.

Perhaps the most interesting observation made in the present study was the vast difference in the capacity of purified phosphoglyceride fractions to support ion transport. Thus, cardiolipin was found to be much more active than the other phosphoglycerides, whereas phosphoglycerides, which would be net neutral at physiological pH, were essentially inactive. The low activity exhibited by the neutral phosphoglycerides possibly can be explained by the presence of impurities, e.g. acidic phosphoglycerides. The exact basis for the difference in capacity of the individual phosphoglyceride must await further experimentation to establish the mechanism responsible for ion transport by phosphoglycerides in the U-tube system.

Is Transport by Phosphoglycerides a Meaningful Physiological Phenomenon?

An important point to be considered is how membranes can be selectively permeable if phosphoglycerides have the capacity to support rather nonspecific transport and countertransport. This consideration argues that phosphoglycerides could not be involved in ion transport at the level of biological membranes. If this is the case, how are phosphoglycerides controlled from participating in countertransport? Obviously, there is a need to study in detail the specificity of the transport stimulated by various phosphoglycerides. In this context, it is interesting to note that some specificity in the binding of monovalent cations has been observed for anionic lipids. Abramson et al. (21) and Breyer (22) have demonstrated a greater relative affinity (about 2:1) for K^+ over Na^+ by the sulfate group of sulfatides. On the other hand, phosphatidic acid and serine phosphoglyceride bind Na^+ in some preference to K^+ (21,23).

The transport of cations and anions by phosphoglycerides and ionophores took place at a slow rate in the system used in this study. The membrane phase used was some 20 cm in thickness, in contrast to 75 ± 25 Å biological

membranes. In addition, phosphoglycerides comprise 20-55% of the dry wt of biological membranes, yet were active in the membrane phase of the U-tubes at low concentrations. Clearly, there seems some justification for considering the notion that phosphoglycerides can play an active role in determining membrane permeability to charged molecules.

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REFERENCES

1. Danielli, F.J., and H. Davson, J. Cell. Comp. Physiol. 5:495 (1935).
2. Hendler, R.W., Physiol. Rev. 51:66 (1971).
3. Robertson, J.D., J. Biophys. Biochem. Cytol. 3:1043 (1957).
4. Brierley, G.P., A.J. Merola and S. Fleischer, Biochim. Biophys. Acta 64:218 (1962).
5. Bulos, B., and E. Racker, J. Biol. Chem. 243:3901 (1968).
6. Bangham, A.D., M.M. Standish and J.C. Watkins, J. Mol. Biol. 13:238 (1965).
7. Hokin, L.F., and M.R. Hokin, Annu. Rev. Biochem. 32:553 (1963).
8. Blondin, G.A., A.F. DeCastro and A.E. Senior, Biochem. Biophys. Res. Commun. 43:28 (1971).
9. Rouser, R., and S. Fleischer, Methods Enzymol. 10:385 (1967).
10. Harris, R.A., and D. Gambal, Anal. Biochem. 5:479 (1963).
11. Rouser, G., G. Kritchevsky, A. Yamamoto, G. Simon, C. Galli and A.J. Bauman, Methods Enzymol. 14:272 (1969).
12. Skipski, F., and M. Barclay, Ibid. 14:530 (1969).
13. Pinkerton, M., L. Steinrauf and P. Dawkins, Biochem. Biophys. Res. Commun. 35:512 (1969).
14. Eiserman, G., S. Ciani and G. Szabo, J. Membrane Biol. 1:294 (1969).
15. Ashton, R., and L.K. Steinrauf, J. Mol. Biol. 49:547 (1970).
16. Pressman, B.C., E.J. Harris, W.S. Jagger and J.H. Johnson, Proc. Nat. Acad. Sci. U.S.A. 58:1949 (1967).
17. Henderson, P.J.F., J.D. McGivan and J.B. Chappell, Biochem. J. 111:521 (1969).
18. Cussler, E.L., D.F. Evans and M.A. Matesich, Science 172:377 (1971).
19. Rosano, H.L., J.H. Schulman and J.B. Weisbeech, Annu. N.Y. Acad. Sci. 92:457 (1961).
20. Schulman, J.H., and H.L. Rosano, in "Retardation of Evaporation by Monolayers," Edited by V.K. LaMer, Academic Press, New York, 1962, p. 97.
21. Abramson, M.B., R. Katzman, R. Curci and C.E. Wilson, Biochem. 6:295 (1967).
22. Breyer, U., J. Neurochem. 12:131 (1967).
23. Kirschner, L.B., Arch. Biochem. Biophys. 68:499 (1957).

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Effects of Ethanol on Membrane Lipids III. Quantitative Changes in Lipid and Fatty Acid Composition of Nonpolar and Polar Lipids of Mouse Total Liver, Mitochondria and Microsomes Following Ethanol Feeding[1]

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ABSTRACT

The effects of ethanol on the total, nonpolar, and polar lipids of whole liver, mitochondria, and microsomes have been evaluated. Differences in the fatty acid composition of various lipid subclasses have been compared in control and ethanol treated mice. On the whole polyunsaturated fatty acids, especially arachidonic (20:4) and docosahexaenoic (22:6), were found to decrease. The significance of an enzymatic mechanism vs. a peroxidative mechanism to explain the results is discussed. Decreases also were observed in the ratios of arachidonate/linoleate following ethanol feeding. These changes are thought to be associated with decreases in the activity of the chain elongation-desaturation system.

INTRODUCTION

Both the size and the number of rat liver mitochondria have been shown to be affected by ethanol (1). In addition changes in fatty acid composition have been associated with membrane stability (2-4). Thus, it would seem that ethanol may have an effect on membrane structure and function, and this effect may be on changes in lipid composition. Liver triglyceride (TG) levels are reported to be influenced by the amount of ethanol given and the duration of exposure (5,6). On the other hand, Fallen et al. (7), found no increase in liver TG following the administration of ethanol. French et al. (8), studied the effects of ethanol on the important membrane constituents cholesterol

[1]The research described herein was conducted while the authors were associated with the University of Detroit, Detroit, Mich. and represents part of the work submitted by J.N. Miceli in partial fulfillment of the requirements for the Ph.D. degree.

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TABLE I

Per Cent Distribution of Nonpolar Lipid and Polar Lipid in in Total Liver, Mitochondria, and Microsomes, of Control[a] and Ethanol Fed Mice[b]

	Total liver		Mitochondria		Microsomes	
Lipid	Control	Alcohol	Control	Alcohol	Control	Alcohol
NPL						
TG	60.4	38.6	48.7	33.8	40.5	11.9
DG	11.7	10.6	22.6	16.8	14.1	13.0
CE	21.6	45.4	22.3	46.4	40.4	64.1
C	5.6	4.3	5.1	1.9	4.0	7.6
FFA	0.7	0.8	1.1	1.0	1.0	2.3
Remainder	—	0.3	0.2	0.1	—	1.1
PL						
CPG	49.2	46.4	63.8	65.5	43.1	35.7
EPG	26.6	21.8	28.2	19.6	42.7	18.2
DPG	8.8	16.1	0.8	2.6	4.2	31.4
SPG	5.1	2.3	0.8	3.5	3.3	4.9
CLPG	4.0	7.8	2.2	0.7	1.8	5.9
SM	3.6	1.1	1.1	4.0	2.3	2.8
Remainder	2.7	4.1	1.1	4.1	2.6	1.1

[a]Values taken from Ref. 19.

[b]NPL = nonpolar lipid, TG = triglyceride, DG = diglyceride, CE = cholesterol esters, C = cholesterol, FFA = free fatty acids, PL = polar lipid, CPG = choline phosphoglyceride, EPG = ethanolamine phosphoglyceride, DPG = diphosphatidyl glycerol (cardiolipin), SPG = serine phosphoglyceride, CLPG = choline lysophosphoglyceride, and SM = sphingomyelin.

TABLE II

Lipid Content of Total Liver Mitochondria and Microsomes from Control and Ethanol Fed Mice
Lipid Wt (mg)[a]

Tissue sample	TL	NPL	PL	Remainder[b]
Total liver				
Control	1549	805	720	24
Alcohol	1747	1200	540	7
Wt change	+198	+395	-180	-17
Per cent of total	+12.8	+25.5	-11.6	-1.1
Mitochondria				
Control	500	190	245	65
Alcohol	383	214	147	22
Wt change	-117	+24	-98	-43
Per cent total	-23.4	+4.8	-19.5	-8.6
Microsomes				
Control	416	174	211	31
Alcohol	316	133	172	11
Wt change	-100	-41	-39	-20
Per cent of total	-25.1	-9.8	-9.4	-4.8

[a]Wt are the total wt obtained from each group of 20 mice. TL = total lipid, NPL = nonpolar lipid, and PL = polar lipid.

[b]Results from less than 100% recovery from column separations.

(C) and cholesterol esters (CE). No differences were found between control and ethanol fed animals.

In animals fed ethanol for 21 days, Fallen et al. (9) found no change in total liver polar lipids or ethanolamine phosphoglyceride (EPG), while Lieber et al. (10) observed increases in both of these under similar conditions.

Recently, evidence was presented for a decrease in the polyunsaturated fatty acids of rat liver mitochondria lipids following acute ethanol intoxication (11). Earlier findings (12) suggested that in ethanol-treated rats a peroxidative decomposition of liver mitochondria lipids may have occurred. A decrease in polyunsaturated fatty acids could be indicative of an in vivo peroxidative breakdown, since these acids would be most susceptible to this mechanism.

One cannot exclude the possibility of increased phospholipase activity following ethanol treatment. Recently evidence was presented showing an increase in plasmalogenase activity in mouse liver after ethanol feeding (13). Other data (14) suggesting that the polar lipid composition did not change after feeding ethanol would argue against the phospholipase hypothesis. However, in an earlier paper we

TABLE III

Effects of Ethanol on the Milligrams of Major Nonpolar Lipids and Polar Lipids in Mouse Liver[a]

	NPL			PL		
Tissue sample	DG	TG	CE	EPG	CPG	DPG
Total liver						
Control	94	488	174	196	354	63
Alcohol	127	463	545	118	250	87
Wt change	+33	-25	+369	-78	-104	+24
Per cent of total	+2.1	-1.6	+23.8	-5.0	-6.7	+1.5
Mitochondria						
Control	43	93	42	69	156	
Alcohol	36	72	99	29	96	
Wt change	-7	-21	+57	-40	-80	
Per cent of total	-1.4	-4.2	+11.4	-8.0	-16.0	
Microsomes						
Control	25	71	70	87	89	9
Alcohol	17	16	85	31	62	54
Wt change	-8	-65	+15	-56	-17	+45
Per cent of total	-1.9	-15.6	+3.6	-13.5	-4.1	+10.8

[a]Values given are for each group of 20 mice. Only lipids which represented 10% or more of either subfraction are considered. See footnote b, Table I for abbreviations.

TABLE IV

Changes in the Milligrams of Fatty Acids in Mouse Total Liver Lipids[a]

Fatty acid		CE	DG	TG	EPG	CPG	DPG	Totals
Saturated								
14:0	C		7			18	3	28
	A		10			10	7	27
	Per cent change[b]		+3.2			-2.2	+6.3	0[c]
16:0	C	49	18	97	59	66	8	297
	A	68	32	85	23	42	17	267
	Per cent change	+10.9	+14.9	-2.4	-18.4	-6.8	+14.3	-1.9
18:0	C		5	19	33	25	3	89
	A		32	41	18	75	8	174
	Per cent change		+28.7	+4.5	-7.6	+14.1	+7.9	+5.5
20:0	C		7					7
	A		0					0
	Per cent change		-7.4					-0.4
Unsaturated								
16:1	C	13	4	41		18	6	82
	A	39	8	16		5	3	71
	Per cent change	+14.9	+4.2	-5.1		-3.7	-4.8	-0.7
18:1	C	60	31	155	32	76	8	362
	A	250	30	135	8	50	7	480
	Per cent change	+109.2	-1.1	-4.1	-12.2	-7.3	-1.6	+4.7
18:2	C	20		128	10	7	29	194
	A	85		117	19	10	36	267
	Per cent change	+37.3		-2.2	+4.6	+0.8	+11.1	+4.7
20:4	C		10		37	75	5	127
	A		3		20	39	2	64
	Per cent change		-7.4		-8.7	-10.2	-4.8	-4.1
22:6	C		9		10	32		51
	A		0		6	0		6
	Per cent change		-9.6		-2.0	-9.0		-2.9

[a]Values given are for each group of 20 mice and represent the average of four experiments. C = control, A = alcohol; see footnote b, Table I for other abbreviations.

[b]Relative to the total amount (from Table III) of lipid subclass in question, i.e., cholesterol esters, diglyceride, etc. present in the control group. Only fatty acids representing 5% or more from each group are considered.

[c]The percentages given in this column are relative to the control total lipid values from Table II.

suggested increased enzymatic hydrolysis of polar lipids to explain the results of incorporation of palmitate-1-^{14}C in mouse liver subcellular fractions (15).

In this article we examine the effects of chronic ethanol consumption on the levels of various nonpolar and polar lipids from mouse liver and also investigate changes in the composition of fatty acids associated with these lipids. Additional evidence in favor of the enzyme hydrolysis hypothesis will be presented by showing that the fatty acid composition of individual lipid classes is altered following ethanol ingestion for 21 days. These lipid changes and alterations in fatty acids were observed in the total liver, as well as the mitochondria and microsomes.

MATERIALS AND METHODS

Animals

Male Swiss-Webster mice weighing 24-26 g were used in this study. The control and alcoholic groups were maintained for 21 days on water and ethanol, respectively, as described previously (13); each group consisted of 20 animals. Subcellular fractions were prepared by ultracentrifugation (16). Histological comparisons, between sections of liver from control and alcohol groups, using Safranin and Sudan Black stains (17) showed that fatty livers had not been produced in the alcoholic mice.

Lipid Extraction and Chromatography

The procedures for lipid extraction, separation into nonpolar and polar lipid fractions, and thin layer chromatography to obtain individual lipid subclasses, are described elsewhere (15,18). Quantitative lipid analysis was carried out, as previously described (19).

Gas Chromatography

Lipid subclasses representing 10% or more of the nonpolar lipid (NPL) and polar lipid (PL) were subjected to transesterification (20). Methyl esters were analyzed qualitatively by gas

TABLE V

Changes in the Milligrams of Fatty Acids in the Mitochondrial Lipids of Mouse Liver[a]

Fatty acid		CE	DG	TG	EPG	CPG	Totals
Saturated							
14:0	C	1	5		7	12	25
	A	8	3		2	5	18
	Per cent change[b]	+16.7	-4.6		-7.2	-4.5	-1.4[c]
16:0	C	12	9	23	16	29	89
	A	10	10	10	7	19	58
	Per cent change	-4.8	+2.3	-14.0	-13.0	-6.4	-6.2
18:0	C		0.4	1	12	10	23
	A		9	13	3	27	52
	Per cent change		+20.0	+12.9	-13.0	+10.9	+5.8
20:0	C					8	8
	A					0	0
	Per cent change					-5.1	-1.6
Unsaturated							
16:1	C	1	1	6	0	11	19
	A	7	3	0	2	0.2	12
	Per cent change	+14.3	+4.6	-6.4	+2.9	-6.9	-1.4
18:1	C	16	15	40	9	51	131
	A	48	7	22	3	14	97
	Per cent change	+76.2	-18.6	-19.3	-8.7	-23.7	-6.8
18:2	C		2	19	2	8	31
	A		0	14	4	7	25
	Per cent change		-4.6	-5.4	+2.9	-0.6	-1.2
20:4	C		4	2	15	5	26
	A		0	1	6	13	20
	Per cent change		-9.3	-1.1	-13.0	+5.1	-1.2
20:6	C		4		4	19	27
	A		0		1	0	1
	Per cent change		-9.3		-4.3	-12.2	-5.2

[a]Values given are for each group of 20 mice and represent the average of four experiments. C = control, A = alcohol; see footnote b, Table I for other abbreviations.

[b]Relative to the total amount (from Table III) of lipid subclass in question, i.e., cholesterol esters, diglyceride, etc. present in the control group. Only fatty acids representing 5% or more from either group are considered.

[c]The percentages given in this column are relative to the control total lipid values from Table II.

liquid chromatography (GLC). Quantitative calculations were made using values for the various lipid classes present in normal mice, which we previously presented (19), and the values for ethanol fed mice reported in this paper (see Table I). GLC analysis was carried out using a Packard dual column Model 7800 instrument equipped with a hydrogen flame detector. A 6 ft glass column of either 14.5% ethylene glycol succinate-methyl silicone polymer (EGSS-X) coated on 100-120 mesh Gas Chrome P or 16% Apiezon M on 60-80 mesh Gas Chrome S was used. Samples were run isothermally at 190 C. The carrier gas was nitrogen at an inlet pressure of 32 psi and a flow rate of 140 ml/min. Peak areas were calculated as the product of peak ht and the width at half peak ht. Percentages are given in terms of peak areas. Corrections for the detector response to the various fatty acids have been incorporated in the percentages. Fatty acid methyl esters were identified by retention times relative to methyl stearate (18:0) and by cochromatography with known standards.

RESULTS

The overall effects of ethanol on the lipids of the total liver and subcellular fractions are given in Table II. These data show that ethanol fed mice had 12.8% more lipid in the total liver than the controls and that this resulted from an increase in NPL exceeding the loss in PL. Mitochondria, on the other hand, showed a loss of 23.4% total lipid (TL) which can be attributed to losses in PL. Microsomes also showed an overall loss of lipid (25.1%), and this resulted from losses of both NPL and PL.

Table III shows that, in the total liver, the increase in NPL was due principally to CE which increased 300% (24% of the TL) and the loss of PL can be attributed to choline phosphoglyceride (CPG) and EPG, the losses in both cases representing more than 5% TL. The loss of mitochondrial and microsomal PL also

TABLE VI

Changes in Milligrams of Fatty Acids in the Microsomal Lipids of Mouse Liver[a]

Fatty acid		CE	DG	TG	EPG	CPG	DPG	Totals
Saturated								
14:0	C		1		7		0.4	8
	A		1		1		4	6
	Per cent change[b]		0		-6.9		+40.0	-0.4[c]
16:0	C	14	5	12	23	16	1	71
	A	16	3	4	7	0	10	40
	Per cent change	+2.8	+8.0	-11.3	-18.4	-18.0	+100	-7.4
18:0	C		1	6	24	9	0.2	40
	A		3	0	5	20	3	31
	Per cent change		+8.0	-8.4	-21.8	+12.3	+31.1	-2.2
20:0	C		4					4
	A		0					0
	Per cent change		-16.0					-1.0
Unsaturated								
16:1	C	6	1	5		0	1	13
	A	6	1	1		16	4	28
	Per cent change	0	0	-5.6		+18.0	+33.3	+3.6
18:1	C	28	8	23	13	11	1	84
	A	33	5	8	4	4	4	58
	Per cent change	+7.1	-12.0	-21.1	-10.3	-7.9	+33.3	-6.2
18:2	C	10		19	4	9	4	48
	A	13		2	5	0	20	40
	Per cent change	+4.3		-23.9	+1.1	-10.1	+17.8	-1.9
20:4	C		2		6	21	1	30
	A		0		6	10	1	17
	Per cent change		-8.0		0	-12.3	0	-3.1
22:6	C		2			12		14
	A		0			0		0
	Per cent change		-8.0			-13.5		-3.4

[a]Values given are for each group of 20 mice and represent the average of four experiments. C = control, A = alcohol; see footnote b, Table I for other abbreviations.

[b]Relative to the total amount (from Table III) of lipid subclass in question, i.e., cholesterol esters, diglycerides, etc. present in the control group. Only fatty acids representing 5% or more from either group are considered.

[c]The percentages given in this column are relative to the control total lipid values from Table II.

was due to CPG and EPG. Diphosphatidyl glycerol (cardiolipin) (DPG) increased in all cases, although in mitochondria this lipid constituted less than the selected 10% cutoff value (Table I). The surprisingly high percentage of DPG in the total liver and microsomes is not understood. The ester-phosphorous ratio (19) of this isolated lipid indicated a relatively pure fraction; and, therefore, the per cent values are thought to be real. Perhaps mouse liver is different with respect to DPG than other tissues analyzed to date. TG decreased in the total liver and subcellular fractions, with the greatest change occurring in the microsomes (15% TL).

Tables IV-VI give the amounts of fatty acids present in the major lipid subclass. Several observations stand out and deserve noting. The amount of arachidonic (20:4) and docosahexaenoic acids (22:6) decreased in all lipids containing them in the total liver, mitochondria, and microsomes of the ethanol treated mice, except in the CPG of mitochondria where 20:4 increased. No particular trend is evident in the changes with regard to linoleic acid (18:2). Overall we see that this fatty acid increased by ca. 5% TL in the total liver and decreased by 1-2% in the mitochondria and microsomes respectively. Since the changes in 18:2 do not appear to be localized, the effect of ethanol to decrease the ratios of 20:4/18:2 (Table VII) is probably a general one rather than a specific effect on any particular lipid. Table IV shows that the large increase in CE in the total liver and mitochondria is primarily oleic acid (18:1).

DISCUSSION

The peroxidative hypothesis to explain decreases in polyunsaturated fatty acids following ethanol ingestion would appear to have little support in view of the work of others (11,21). Thus it would appear that the effect is more on the enzyme level, as we suggested earlier (15). Evidence has been presented suggesting that the characteristic fatty acid composition of phospholipids is determined by redistribution of the fatty acids after the nitrogenous base has been

attached (22). The activity of the fatty acyl-CoA:lysophosphatide transferases catalyzing this reaction recently has been shown to be enhanced with increased ethanol consumption (23). Thus, a mechanism of this type seems more likely than peroxidation. This is supported further when one examines the data in Tables V and VI. Here we see that, in both the mitochondria and microsomes of alcohol treated mice, a change has occurred in the fatty acid distribution of the major lipids. Our recent studies on the effects of ethanol on aldehydogenic lipids (13) would also support this finding.

Examination of Tables IV-VI also reveals that significant increases in the stearic acid (18:0) content of various lipids has occurred in the total liver and mitochondria of ethanol fed mice. This is in contrast to others (11) who observed decreases in this fatty acid in rats fed ethanol. The reason for this discrepancy is not understood; however, it could be related to experimental conditions, most notably diet and the time duration of the experiments. Corn oil-fed rats and coconut oil-fed rats gave opposite results with respect to fatty acids found in the CPG and EPG following ethanol treatment (23).

A decrease in the arachidonate-linoleate ratio of liver lipids following alcohol ingestion also has been observed by others (24). Similar changes in heart lipids also were reported (25). Such decreases have been suggested to account for membrane stability (3) and mitochondria fragility (2). It has been suggested (26) that the decrease observed in liver mitochondria was due to decreases in the activity of the chain elongation-desaturation system which converts linoleate to arachidonate. Since liver CoA content is decreased markedly by ethanol (27), a decrease in the activity of the enzyme system seems a logical explanation.

TABLE VII

Effects of Ethanol on the Arachidonate-Linoleate Ratios in Lipids of Mouse Total Liver, Mitochondria, and Microsomes

Lipid fraction	Total liver	Mitochondria	Microsomes
Total lipids			
Control	0.65	0.90	0.62
Alcohol	0.24	0.69	0.42
Change	-0.41	-0.21	-0.20
Nonpolar lipids			
Control	0.67	0.28	0.07
Alcohol	0.01	0.07	0.00
Change	-0.66	-0.21	-0.07
Polar lipids			
Control	2.43	2.20	1.47
Alcohol	0.94	1.30	0.68
Change	-1.49	-0.90	-0.79

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REFERENCES

1. Kiessling, K.H., and L. Pilstrom, Acta Pharm. Tox. Kbh. 24:103 (1966).
2. DePury, G.G., and F.D. Collins, Chem. Phys. Lipids 1:1 (1966).
3. Hyashida, T., and O.W. Portman, J. Nutr. 81:103 (1963).
4. Holmer, G., and B. Tronier, Lipids 7:534 (1972).
5. Klastskin, G., H.M. Gewin, and W.A. Krehl, Yale J. Biol. Med. 23:317 (1951).
6. Mallov, S., Proc. Soc. Exptl. Biol. Med. 88:246 (1955).
7. Fallon, H.S., P.M. Gertman, and E.L. Kemp, Biochem. Biophys. Acta 187:94 (1969).
8. French, S.W., T.J. Ihrig, and R.J. Morin, Quart. J. Stud. Alc. 31:801 (1970).
9. Fallon, H.S., L.A. Persch, and G. Klatskin, Biochem. Biophys. Acta 98:470 (1965).
10. Lieber, C.S., D.P. Jones, and L.M. DeCarli, J. Clin. Invest. 44:1009 (1965).
11. Comporti, M., E. Burdino, and F. Raja, Life Sci. 10:885 (1971).
12. DiLuzio, N.R., and A.D. Hartman, in "Biological and Clinical Aspects of Alcohol Metabolism," Edited by V.M. Sardesai, Charles C Thomas, Springfield, Ill., 1969, p. 133.
13. Ferrell, W.J., and J.N. Miceli, Comp. Biochem. Physiol. 41B:19 (1972).
14. Kiessling, K.H., and C.G. Lundquist, Scand. J. Clin. Lab. Invest. 18(Suppl. 92):80 (1966).
15. Ferrell, W.J., and J.N. Miceli, Quart. J. Studies on Alc. 31:810 (1970).
16. Hogeboom, G.H., W.C. Schneider, and G.E. Pallade, J. Biol. Chem. 172:619 (1948).
17. Barka, T., and P.J. Anderson, "Histochemistry Theory, Practice, and Bibliography," Harper and Row, New York, N.Y., 1965, p. 120.
18. Gilbertson, J.R., W.J. Ferrell, and R.A. Gelman, J. Lipid Res. 8:38 (1967).
19. Miceli, J.N., and W.J. Ferrell, Physiol. Chem. Phys. 4:131 (1972).
20. Morin, R.J., S. Bernick, J. Mead, and R.B. Alfin-Slater, J. Lipid Res. 3:432 (1962).
21. Hashimoto, S., and R.O. Recknagel, Expt. Mol. Pathol. 8:225 (1968).
22. Lands, W.E.M., and P. Hart, J. Biol. Chem. 240:1905 (1965).
23. Mendenhall, C.L., R.H. Bradford, and R.H. Furman, Biochem. Biophys. Acta 187:510 (1969).
24. French, S.W., and R.J. Morin, in "Biochemical and Clinical Aspects of Alcohol Metabolism," Edited by V.M. Sardesai, Charles C Thomas, Springfield, Ill., 1969, p. 123.
25. Reitz, R.C., H. Helsabeck, and D.P. Mason, Lipids 8:80 (1973).
26. Shaw, G.P., S.W. French, T.J. Ihrig, T.T. Tonaka, and R.J. Morin, Lab. Invest. 24:447 (1971).
27. Ammon, H.P.T., C.-j. Estler, and F. Heim, Biochem. Pharmacol. 18:29 (1969).

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Lipid Labeling with ^{32}P-Orthophosphate and ^{14}C-Acetate in Marine Copepods

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ABSTRACT

Freshly collected *Calanus pacificus* were maintained in sea water containing 25 μCi/ml [^{32}P]orthophosphate or 1 μCi/ml [^{14}C]acetate at 10 C for 24 hr. The animals took up label from the environment and incorporated it into various lipid fractions. After incubation with [^{14}C]acetate the order of specific activity of the different lipid classes was: phospholipids > free fatty acids > wax esters > triglycerides. Argentation thin layer chromatography of the fatty acid methyl esters showed that ca. 50% of the activity was in saturated fatty acids and 34% in polyunsaturated acids. When the animals were exposed to [^{32}P]orthophosphate, lysophosphatidyl choline became most heavily labeled, followed by lysophosphatidyl ethanolamine, sphingomyelin, phosphatidyl ethanolamine, and phosphatidyl choline. Comparison of the data obtained with those available for decapods and mammals revealed striking similarities between these phylogenetically distant groups. It is believed that labeling the lipids of marine and freshwater planktonic crustaceans in this way will provide much information about the metabolism of lipids in these organisms.

INTRODUCTION

Marine copepods are one of the most important links between the photosynthetic organisms and fishes in the oceans. It is evident that the metabolism of lipids in these organisms is of great importance in determining the lipid types and compositions in higher trophic levels. However, little is known about lipid metabolism in copepods, because of the difficulties of maintaining them in the laboratory for long periods of time. In feeding experiments Lee, et al., (1) showed that *Calanus helgolandicus* incorporates the dietary fatty acids largely unchanged into its wax esters, while fatty acids of phospholipids were less affected by changes in the type of food. Petipa (2,3) has demonstrated that *C. helgolandicus* utilizes fat during starvation and vertical migration; and Lee, et al., (4) has shown that the observed decrease in the lipid content of starving animals is due to catabolism of triglycerides and wax esters.

Isotopic labeling would be extremely useful in studying the lipid metabolism of these animals, but their small size does not permit the use of the conventional injection techniques in vivo nor the preparation of slices from different organs for in vitro incubation methods. In an attempt to overcome these difficulties, we started from the observations of Stephens (5), Johannes (6), Taylor (7), Shuliene (8), and Testerman (9) showing that marine invertebrates are able to take up inorganic and small organic molecules from the environmental medium and incorporate them into various body components, such as proteins or lipids. This paper describes labeling with [^{32}P]orthophosphate and [1-^{14}C]acetate and some aspects of the metabolism of neutral and phospholipids in a marine copepod, *Calanus pacificus*

MATERIALS AND METHODS

C. pacificus (copepodid V's and adult females; 2.5 mm average size) were collected in Saanich Inlet, Vancouver Island, British Columbia, Canada, in early July 1972 from a depth of 50-100 m. The animals were transferred into filtered sea water and kept in a refrigerator at 10 C. Twenty individuals were used for the ^{32}P and 38 for [^{14}C]acetate labeling experiments. They were held for 24 hr in beakers containing 25 ml filtered sea water and 25 μCi/ml [^{32}P]orthophosphate or 1 μCi/ml [1-^{14}C]acetate. At the end of the experiment the labeled water was pipetted off, and the animals were rinsed three times with sea water containing either 0.1M Na_2HPO_4 or sodium acetate. Total lipid was extracted with chloroform:methanol 2:1 and the extract solution separated into two phases by adding 0.2 volumes of 0.1 M potassium chloride. The chloroform phase was washed six times with Folch upper phase containing either 0.1 M Na_2HPO_4 or 0.1 M sodium acetate. Aliquots of the chloroform phase were counted; other aliquots were separated on precoated Silica Gel

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G plates (E. Merck, Darmstadt, West Germany, supplied by Brinkmann Instruments Inc.) into wax ester, triglycerides, free fatty acid, diglyceride plus free sterol, and phospholipid fractions using petroleum ether: diethyl ether: acetic acid (75:25:1) as solvent. From another aliquot sterol esters and wax esters were separated on MgO plates (10) using petroleum ether: diethyl ether: ethyl acetate (50:50:1). Authentic wax esters and cholesterol esters were used as standards. The phospholipids and free fatty acids remaining at the origin of MgO plates were transmethylated in the presence of the MgO with methanol containing 1% sulphuric acid and subjected to argentation thin layer chromatography (TLC) on Silica Gel G plates containing 10% $AgNO_3$. The solvent system was 25% diethyl ether in petroleum ether. Phospholipids were separated by two-dimensional TLC, according to Parsons and Patton (11). Visualization was made either by exposing the plates to iodine vapors or spraying with Rhodamine 6-G. The adsorbent from each spot was transferred to a vial and counted in a liquid scintillation spectrometer (Beckman Model LS-100).

The lipid content of the animals was determined gravimetrically by extracting 1200 specimens. Dry wt was determined by measuring the wt of 200 animals fixed in formaldehyde. Composition of the extracted lipid was determined densitometrically after thin layer separation, using the method of Fewster, et al., (12). The phospholipid compositions were determined quantitatively from the two dimensional TLC separations measuring the phosphorus content of each spot by the method of Kahovcova and Odavic (13).

Sufficient label was incorporated into lipid by this technique to permit using a single copepod to obtain useful values for the distribution of radioactivity in various lipid types. One animal was placed at the origin of a TLC plate and crushed; after drying, the plate was developed in the usual way. The separated zones then could be located by spraying with Rhodamine G, scraped off, and counted.

RESULTS AND DISCUSSION

This article reports the ability of *C. pacificus* to take up inorganic phosphate and sodium acetate from the environment and incorporate them into various lipid fractions. The rate of uptake of ^{32}P – and presumably of ^{14}C also – remained appreciable over 24 hr, as shown in Figure 1. Some successful exploratory experiments also were carried out with a euphausid, *Thysanoesa raschii*; again, useful labeling of total lipids occurred. Labeling lipids in this way

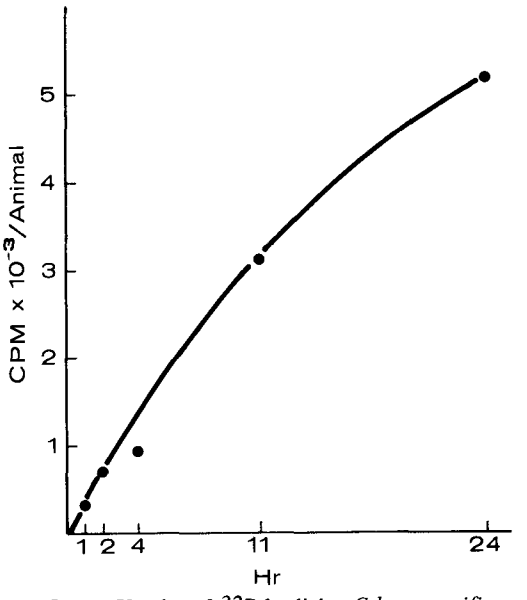

FIG. 1. Uptake of ^{32}P by living *Calanus pacificus*. Individual animals were counted after incubation for the specified time in 25 ml sea water containing 25 μCi [^{32}P] orthophosphate.

will facilitate the study of fat metabolism in planktonic crustaceans under various experimental conditions.

The average dry wt of the animals was 93.6 μg, and the fat content was 19.2%. Table I shows that ca. half of the lipid present in *C. pacificus* is wax esters; phospholipids are the next most abundant component, followed by triglycerides. After exposing the animals to [1-^{14}C]acetate, the specific activity of the total lipids was 2.4 x 10^5 cpm/mg lipid; all the lipid fractions were labeled. Ca. 50% label was recovered in the phospholipids, 25% in a fraction with the R_f of sterol esters on MgO, and ca. 14% in free fatty acids. The fact that the percentage of the activity in the free fatty acid fraction was so low suggests that fatty acids formed from acetate were esterified rapidly to form more complex lipids. The observed order of specific activity of these compounds was: phospholipids > free fatty acids > wax esters > triglycerides. The higher specific activity of the wax ester fraction over the triglycerides agrees with the observations that most calanoid copepods store wax esters instead of triglycerides (14) and that triglycerides are catabolized preferentially during starvation (4). Unfortunately, the present investigations do not say anything about the extent of the conversion of fatty acids into fatty alcohols; but in similar studies on *C. helgolandicus*, we found ca. 40% radioactivity of the wax esters in

TABLE I

Calanus pacificus Lipids: Composition and [^{14}C] Acetate Incorporation[a]

Lipid class	Composition		^{14}C Incorporation	
	Weight %	nMoles[b]/animal	cpm/animal	cpm/nMole
Wax esters	48.5	17.7[d]	172	9.72
Sterol esters[c]		ND	846	ND
Triglycerides	16.6	5.06	21.5	4.23
Free fatty acids	5.1	3.2	589	184
Diglycerides	ND[e]	ND	355	ND
Free sterols[f]	5.0	2.43	117	48
Phospholipids	24.4	6.06	2,180	360

[a]Animals were incubated for 24 hr at 10 C in sea water containing 1 μCi/ml sodium [1-^{14}C] acetate.

[b]Wax esters were assumed to be n-hexadecyl oleate (M.W. 506). Mol wt of glycerides were calculated assuming that palmitic acid esterified carbon atoms 1 and 3, with docosahexaenoic acid occupying position 2. The total phospholipids were calculated as 1-palmitoyl-2-docosahexaenoyl phosphatidyl choline.

[c]Fraction had same R_f as cholesterol ester.

[d]Assuming wt of sterol esters to be negligible.

[e]ND = not determined.

[f]Fraction had same R_f as cholesterol.

the alcohol moieties.

From the data of Table I it is evident that most of the ^{14}C-activity was incorporated into fatty acids. Separation on silver nitrate plates of the fatty acid methyl esters obtained from the phospholipid and free fatty acid fractions showed that saturated fatty acids contained about 49% total radioactivity, followed by polyunsaturated acids (34%), and monounsaturated acids (17%). In marine crustaceans the amounts of linoleic and linolenic acids are usually low; consequently, most of the activity found in the polyunsaturated fraction should be in C_{20} and C_{22} polyenoic fatty acids. The bulk of these long chain polyunsaturated fatty acids in epipelagic marine copepods is unlikely to result from direct deposition of dietary acids; rather they probably are biosynthesized by the copepods from dietary C_{18} polyenoic acids via chain elongation and desaturation (15).

After 24 hr the specific activity observed for the total [^{32}P] phospholipids was 4 x 10^5 cpm/mg, and all spots separated by two dimensional TLC were labeled. Lysophosphatidyl choline had the highest specific activity, followed by lysophosphatidyl ethanolamine, sphingomyelin, phosphatidyl ethanolamine, and phosphatidyl choline (Table II). The data suggest that the metabolism of phospholipids in *C. pacificus* is, in some respects, comparable to that in higher crustaceans or mammals. Phosphatidyl choline was more heavily labeled than phosphatidyl ethanolamine after exposure of *C. pacificus* to [^{14}C] acetate, just as observed for lobsters and mouse brain preparations (16,17). On the other hand, in agreement with observations made on lobster (16) and mammals (18-21), *C. pacificus* accumulated more label from exogenous [^{32}P] orthophosphate into phosphatidyl ethanolamine than into phosphatidyl choline. Published data from rat liver suggest that incorporation of [^{32}P] orthophosphate into different phospholipids varies with the degree of unsaturation of the esterifying fatty acids (22). For instance, the following order of specific activity of the various molecular species of [^{32}P] phosphatidyl choline was obtained: dienoic > monoenoic > hexaenoic > tetraenoic. In the case of phosphatidyl ethanolamines the observed order was: hexaenoic > tetraenoic > dienoic > monoenoic. Because of the limited amount of lipid available in this study, no detailed analysis of fatty acid composition was made; however, in the closely related species *Calanus plumchrus*, phosphatidyl choline contained some 15% more polyunsaturated fatty acids (including 4.4% arachidonic acid) than did phosphatidyl ethanolamine (23). Similar values for *C. pacificus* could explain the lower specific activity we observed for [^{32}P] phosphatidyl choline compared to phosphatidyl ethanolamine.

To understand the relative specific activities of the lyso- and diacyl-phospholipids, we need to consider their biosynthetic pathways. Assuming that the major pathways established in mammals also prevail in crustaceans, then the lyso-compounds are formed exclusively from the corresponding (diacyl) phosphatidyl ethanolamine or phosphatidyl choline; therefore, ^{14}C or ^{32}P essentially can be introduced into

TABLE II

Composition and Radioactivity of Phospholipids of *Calanus pacificus*[a]

Phospholipid	Wt % of total P[b]	nMole/animal[c]	Cpm/animal		Cpm/nMole	
			^{32}P	^{14}C	^{32}P	^{14}C
Lysophosphatidyl choline	0.39	0.031	218	33.5	7,030	1,080
Sphingomyelin	2.30	0.156	136	170.0	872	1,090
Lysophosphatidyl ethanolamine	2.50	0.227	327	38.5	1,440	170
Phosphatidyl choline	41.71	2.22	246	1,732	111	780
Phosphatidyl ethanolamine	37.01	2.12	720	646	324	291
Phosphatidyl inositol	1.23	0.061	34.6	33.0	570	540
Phosphatidyl serine	7.63	0.414	50.8	88.5	123	214

[a]Animals were incubated in sea water in the presence of either 25 μCi/ml [^{32}P]orthophosphate or 1 μCi/ml sodium [1-^{14}C]acetate for 24 hr.

[b]Identical to "mole per cent of total phospholipid" and taken to be an acceptable approximation to the "wt per cent of total phospholipids." Several minor components totaling 8.2% were not investigated further.

[c]Mol wt were calculated assuming that all glycerides were esterified at carbon-1 with palmitic acid and at carbon-2 (except for the lyso-compounds) with docosahexaenoic acid. Palmitic acid was assumed to be the only fatty acid present in sphingomyelin.

the lyso-compounds only by de novo synthesis from an intermediate diglyceride and, for ^{14}C, probably ultimately from phosphatidic acid (rather than triglyceride). Our finding that the ^{32}P specific activities of lysophosphatidyl ethanolamine (LPE) and lysophosphatidyl choline (LPC) are higher than those of the corresponding diacyl derivatives implies small compartments of newly synthesized labeled phosphatidyl ethanolamine and phosphatidyl choline being acted upon by phospholipase A_2 to remove the 2-acyl moiety, forming LPE and LPC. The lyso-compounds are known to be reacylated—here with ^{14}C-fatty acids—to the diacyl derivatives, so there is a second route for the introduction of ^{14}C into phosphatidyl choline and phosphatidyl ethanolamine. LPE and LPC are regarded as important intermediates in determining the individual fatty acid distributions in phospholipids, since they can be re-esterified selectively at the 2 position with polyunsaturated fatty acids to form phosphatidyl ethanolamine or phosphatidyl choline again (22), thus maintaining the appropriate liquid crystalline structure of the membranes which is vital to the life of the copepod. Additional data are necessary to provide a convincing explanation of the observed ^{14}C relative specific activities.

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REFERENCES

1. Lee, R.F., J.C. Nevenzel and G.-A. Paffenhöfer, Mar. Biol. 9:99 (1971).
2. Petipa, T.S., Dokl. Akad. Nauk SSSR 155:470 (1964).
3. Petipa, T.S., Dokl. Ibid. 156:1440 (1964).
4. Lee, R.F., J.C. Nevenzel, G.-A. Paffenhöfer and A.A. Benson, J. Lipid Res. 11:237 (1970).
5. Stephens, G.C., Am. Zool. 8:95 (1968).
6. Johannes, R.E., Limnol. Oceanogr. 9:235 (1964).
7. Taylor, A.G., Comp. Biochem. Physiol. 29:243 (1969).
8. Shuliene, R. Yu., Gidrobiol. Zh. 7:83 (1971).
9. Testerman, J.K., Biol. Bull. 142:160 (1972).
10. Kaufmann, H.P., H.K. Mangold and K.D. Mukherijee, J. Lipid Res. 12:506 (1971).
11. Parsons, J.G., and S. Patton, Ibid. 8:696 (1967).
12. Fewster, M.E., B.J. Burns and J.F. Mead, J. Chromatogr. 43:120 (1969).
13. Kahovcova, J., and R. Odavic, Ibid. 40:90 (1969).
14. Lee, R.F., J. Hirota and A.M. Barnett, Deep Sea Res. 18:1147 (1971).
15. Mead, J.F., Prog. Chem. Fats Other Lipids. 9:161 (1968).
16. Shieh, H.S., Comp. Biochem. Physiol., 30:679 (1968).
17. Kanazawa, I.N., N. Keta and T. Yamakawa, J. Neurochem. 19:1483 (1972).
18. Gurr, M.I., C. Prottery and J.N. Hawthorne, Biochim. Biophys. Acta 106:357 (1965).
19. Marinetti, G.V., J. Ebbland, M. Albrecht and E. Stotz, Ibid. 30:543 (1958).
20. Taylor, C.B., E. Bailey and W. Bartley, Biochem. J. 105:605 (1967).
21. McMurray, W.C., and R.M.C. Dawson, Ibid. 112:91 (1969).
22. Van Golde, L.M.G., G.L. Scherphof and L.L.M. Van Deenen, Biochim. Biophys. Acta 176:635 (1969).
23. Benson, A.A., R.F. Lee and J.C. Nevenzel, in "Current Trends in the Biochemistry of Lipids," Edited by J. Ganguly and R.M.S. Smellie, Academic Press, London, England, 1973, p. 179.

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Alternate Pathways of Cerebroside Catabolism

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ABSTRACT

A search was made for new degradative pathways for glucosyl and galactosyl ceramides in an effort to explain the failure of these lipids to accumulate in the brains of children with Krabbe's or Gaucher's disease. Using various buffers and incubation conditions, we tested brain homogenates from 12 day old rats with the stearate-labeled and galactose-labeled lipids. No evidence for direct deacylation (and formation of psychosines) could be obtained, nor was there any evidence for transacylation of sphingosyl phosphoryl choline or oxidation of the 6 position of the galactose moiety. Two new derivatives of galactosyl ceramide were observed, possibly fatty acid esters of unknown polar compounds. It is tentatively proposed that the etiology of infantile Krabbe's and Gaucher's diseases involves, not an accumulation of galactosyl and glucosyl ceramides, with consequent formation of toxic products, but rather malfunction of some other role of the corresponding glycosidases.

INTRODUCTION

The only known pathway for catabolism of glucosyl and galactosyl ceramides is through two β-glycosidases (1,2). In the case of the galactosidase, the enzyme appears to be quite specific and can be distinguished from a number of other β-galactosidases (1,3,4). It does seem to attack galactosyl sphingosine (5-7), a lipid which hypothetically could be derived from galactosyl ceramide via an amidase. Glucocerebrosidase probably is not the only mammalian β-glucosidase, judging from comparisons between aryl glucoside and ceramide glucoside as substrates (2,8).

In Krabbe's disease (infantile globoid leukodystrophy), the primary genetic lesion appears to be in the cerebroside galactosidase (9). The striking feature of this disease is that there is no accumulation of galactosyl ceramide in brain; but instead there is a marked, fatal lack of brain development. Miyatake and Suzuki (5) have made the suggestion that the galactosyl ceramide is converted to galactosyl sphingosine, which may be highly toxic and thus cause the fatal failure to develop. This alternative catabolic product is assumed to be so toxic that detectable concentrations fail to accumulate. The failure of cerebroside to accumulate does not seem to be due to loss of the synthetic enzymes which make the ceramides and galactosyl ceramides (10).

A similar situation could be postulated in the infantile form of Gaucher's disease, which seems to be due to a severe lack of glucosidase (11). The young brain is an active manufacturer of glucosyl ceramide for use in making gangliosides; and there seems to be an active catabolism of gangliosides, so one should expect the brain to accumulate much glucosyl ceramide in infantile Gaucher's disease. Instead there seems to be no accumulation, and the brain simply fails to mature, degenerating to a fatal condition, as in the Krabbe disorder. (Although a few cases of the infantile form of the disease have been found to accumulate some glucocerebroside in the brain, the lipid appears to be in unusual cells, rather than in neurons [12]). Thus one might suggest that glucosyl ceramide could be converted to glucosyl sphingosine, a potentially highly toxic substance.

While our early studies on galactosyl ceramide breakdown (1) gave negative evidence for fatty acid release (except for a bit that could be attributed to ceramidase action), no such examination appears to have been made in the case of glucosyl ceramide. Consequently we have reopened the question with the aid of cerebrosides labeled in the galactose and fatty acid moieties by incubating them with brain homogenates and examining the radioactive products. No evidence for fatty acid release could be obtained, but some conversion of galactosyl ceramide to two unknown polar substances was observed.

MATERIALS AND METHODS

Substrates

The synthesis of the two cerebrosides labeled with [1-^{14}C]stearic acid has been described before (13,14). Examination of the galactosyl ceramide by thin layer chromatography (TLC) radioautography showed only one spot, and elution of this spot and other plate regions indicated a radiopurity of over 97%. The glucosyl ceramide showed a slight radioactive streak above and below the cerebroside spot and a minor spot just below the main spot,

TABLE I

Fatty Acid Fraction Produced from Incubations with Brain Homogenate and Stearate-Labeled Cerebrosides

Substrate	Buffer	Per cent conversion of labeled substrate: Emulsion (8 mg)	Emulsion (32 mg)	On Celite (32 mg)
Galactosyl ceramide 200,000 cpm	Phosphate 7.0	0.03[a]	0.03[a]	0.04[a]
	Citrate 4.0	.03	.04	.04
	Citrate 5.0	.03	.04	.04
	Citrate 6.2	.03	.06	---
	Phosphate 6.0	.04	.04	.05
	Phosphate 7.0	.04	.05	.04
	Phosphate 8.0	.04	.05	---
	Tris 7.2	.04	.05	---
	Tris 8.0	.06	.06	.05
	Tris 8.9	.05	.04	.05
Glucosyl ceramide[b] 200,000 cpm	Citrate 5.0	1.76[a]		0.20[a]
	Citrate 4.0	1.39		.28
	Citrate 5.0	5.78		.28
	Phosphate 6.0	4.33		.50
	Phosphate 7.0	3.79		.35
	Tris 8.0	1.38		.34
	Tris 8.9	1.42		.34

[a]Data from boiled enzyme. These numbers were not subtracted from the others.

[b]The glucocerebroside used to make the emulsion had not been purified recently and yielded a high blank in the fatty acid fraction.

which could be the dihydrosphingosine form. The radiopurity, taking the minor spot to be an impurity, was 96%. Galactosyl ceramide labeled in the 6 position of the sugar with tritium was made by the borohydride method (15). Sphingosyl phosphoryl choline hydrochloride was made from sphingomyelin by hydrolysis in methanol-water-HCl (16) and purified with a long silica gel column.

The cerebrosides were tested as emulsions in Tween 20, Myrj 59, and Na taurocholate (17); tris oleate was omitted from the emulsion.

Enzyme Test Procedure

Some incubations were performed with 0.4 ml cerebroside emulsion (0.1 mg cerebroside), 0.1 mmole buffer, 0.2 μmoles dithiothreitol, and brain homogenate in a total volume of 1 ml. The homogenate was made from brains of 12-day-old Sprague-Dawley rats in 11 volumes cold water. The homogenate was stored in portions in the freezer, and each series was carried out on material which had been thawed only once. The rats were chosen to be at the pre-myelination age, which corresponds more closely to the human disorders and which minimizes dilution of the radioactive substrate by endogenous cerebroside.

Some incubations were carried out in the absence of detergent, and the cerebroside then was coated onto 50 mg Celite Analytical Filter Aid by evaporation from solution. To this was added 0.1 mmole buffer, 0.2 μmole dithiothreitol, and brain homogenate, as above.

Where sphingosyl phosphoryl choline was added to the mixture, 0.2 mg lipid was applied to the bottom of the incubation tube by evaporation from solution prior to the other additions. The lipid dissolved readily in each buffer.

Buffers used were Na-citrate, Na-phosphate, and Tris-HCl at pH noted in the tables. The tubes were shaken 1 hr at 37 C, then 20 ml chloroform-methanol were added. The mixture was acidified with 4 ml 0.5 M citric acid, and the resultant upper layer was discarded. The lower layer was washed with 10 ml methanol-water (1:1) and filtered to remove protein. The filtrate was evaporated to dryness under vacuum with the aid of benzene additions, taken up in chloroform, and applied to a silica gel column (0.4 x 6 cm, 0.32 g). A fatty acid fraction was eluted with 10 ml chloroform, a cerebroside fraction with 10 ml chloroform-methanol (90:10), and a polar lipid fraction with 10 ml chloroform-methanol (1:2). Portions of the first and third fractions were counted and examined by TLC radioautography.

RESULTS

Examination of the first column fraction

TABLE II

Fatty Acid Fraction Produced from Incubations with Brain Homogenate and Labeled Glucocerebroside in the Presence of Glucosidase Inhibitors

Buffer	Inhibitor	Per cent conversion of cerebroside
Citrate 5.0[a]	None	5.37
	None (boiled enzyme)	1.76
	5 μM hexyl glucosyl sphingosine	2.05
	5 mM δ-gluconolactone	1.94
Phosphate 6.0[b]	None	0.33
	None (boiled enzyme)	.26
	20 μM hexyl glucosyl sphingosine	.17
	10 mM δ-gluconolactone	.22

[a]These incubations run with cerebroside emulsion and 16 mg brain.

[b]These incubations run with cerebroside dispersed on celite and 32 mg brain.

from galactocerebroside incubations showed very slight formation of free fatty acid (Table I). While radioactive ceramide was formed in the incubations containing detergents and taurocholate, there was evidently insufficient ceramide for further conversion at an appreciable speed into free fatty acid. In the case of glucocerebroside, the first column fraction did show an appreciable amount of radioactivity, especially at pH 5 with the emulsified substrate and pH 6 with the nonemulsified substrate. The formation of fatty acid was much less in the tubes lacking taurocholate and detergents, which can be explained by the well-established need for these adjuncts in the normal catabolic pathway (via glucosidase and ceramidase). Evidently glucosidase was sufficiently active in these preparations to raise the ceramide level to the point where ceramidase action could be observed.

Further evidence for this interpretation was obtained by incubating labeled glucocerebroside in the presence of inhibitors of glucosidase (Table II). Both inhibitors used brought down the yield of labeled fatty acid close to the blank level.

Assuming the inhibitors also do not block the hypothetical amidase, we conclude that the radioactivity observed without inhibitors is due to a rapid hydrolysis of glucocerebroside to ceramide, and then to sphingosine and free fatty acid.

The polar lipid fraction (Table III) showed some conversion of galactocerebroside to more polar material, particularly at pH 6 and 8 with emulsions or plain cerebroside. This conversion was greater in tubes containing more homogenate and generally seemed higher in the absence of detergents. Radioautography of a TLC plate (silica gel, chloroform-methanol-water [117:45:8]) showed that this fraction contained a radioactive band at the cerebroside position (R_f 0.61), evidently due to some trailing from the second column eluent, and two bands at R_f 0.52 and 0.38. When this examination was carried out with the same column fraction after alkaline methanolysis at room temperature for 1 hr (18), the cerebroside band was found to be unchanged, but the two lower bands were now even lower, with R_f 0.29 and 0.24. From this it would appear that the two metabolic products were fatty acid esters of some polar cerebroside derivative. The positions of the methanolyzed compounds did not correspond to any known galactosyl ceramide derivatives, including galactosyl galactosyl ceramide. The lower band corresponded to the faster region of cerebroside sulfate or lecithin.

Some conversion of glucocerebroside to polar material also was observed in the absence of detergent (Table III) but was not characterized further, because we assumed that this was a series of ganglioside intermediates made from endogenous sugar donors. The glucosidase inhibitors had no effect on this conversion.

Incubations also were carried out with stearate-labeled galactosyl ceramide and unlabeled sphingosyl phosphoryl choline (lysosphingomyelin) on the theory that the cerebroside is not degraded by an amidase but by a specific fatty acyl transferase which could form galactosyl sphingosine and sphingomyelin. Here too there was some conversion to polar lipid, appearing in the third column eluent, but this was to the same extent as that found without the lysosphingomyelin. TLC radioautography showed the same labeled bands without any sign of radioactivity in the sphingomyelin.

Another possible catabolic route for galactocerebroside was tested by incubations with stearoyl [6-^{3}H]galactosyl sphingosine, dispersed on Celite or emulsified, as described

TABLE III

Polar Lipid Fraction Produced by Incubations with Brain Homogenate and Stearate-Labeled Cerebrosides

Substrate	Buffer	Per cent conversion of labeled substrate		
		Emulsion		On Celite
		(8 mg)	(32 mg)	(32 mg)
Galactosyl ceramide	Phosphate 7.0	0.24[a]	0.36[a]	0.84[a]
	Citrate 4.0	.20	.84	.65
	Citrate 5.0	.22	.58	.61
	Citrate 6.2	.17	1.10	—
	Phosphate 6.0	.32	1.28	2.15
	Phosphate 7.0	.32	.97	1.26
	Phosphate 8.0	.33	.86	—
	Tris 7.2	.31	.65	—
	Tris 8.0	.20	1.62	1.06
	Tris 8.9	.24	.71	1.81
Glucosyl ceramide[b]	Citrate 5.0	2.76[a]		1.42[a]
	Citrate 4.0	1.89		1.72
	Citrate 5.0	1.48		1.71
	Phosphate 6.0	1.74		2.64
	Phosphate 7.0	2.09		1.94
	Tris 8.0	2.12		2.41
	Tris 8.9	1.95		1.50
	Phosphate 6.0			.68[a]
	Phosphate 6.0			1.12
	Phosphate 6.0			1.28[c]
	Phosphate 6.0			1.04[d]

[a]Data from boiled enzyme.

[b]The glucocerebroside used to make the emulsion had not been purified recently and yielded a high blank in the polar lipid fraction.

[c]Incubation included 20 μM hexyl glucosyl sphingosine.

[d]Incubation included 10 mM gluconolactone.

above. After incubating the mixture for 2 hr with brain at pH 6 (phosphate), 7.2 and 8 (Tris), we lyophilized the suspension and measured the radioactivity in the collected water. (In the case of the samples containing Celite, the powder had to be removed before lyophilization to prevent flaking and carryover into the condenser.) In no case was tritium found in the water, indicating a lack of a dehydrogenase that could attack the 6 position of the sugar.

DISCUSSION

Disregarding the obvious dangers of reporting negative findings and of not trying every possible buffer and incubation medium, we conclude that neither glucosyl nor galactosyl ceramide is metabolized by young brain to form the corresponding hexosyl sphingosine. Our finding of hitherto undetected galactocerebroside derivatives of a polar type raises the possibility that these are toxic in the brain of the Krabbe child, and that their accumulation has not been noticed in previous examinations of the brain lipids. More likely, in our opinion, the seriousness of infantile Krabbe's and Gaucher's diseases is due to malfunction of a thus far unknown role of the cerebroside hydrolases. Preliminary evidence with a glucosidase inhibitor and cultured brain tumor cells suggests a role in membrane transport (19).

The observations that hexosyl sphingosines can be formed enzymatically from the free base and UDP-Hexose (20,21) and that the hexosyl sphingosines can be hydrolyzed to free base and hexose are attributed to nonspecificity on the part of the cerebroside synthetases and hydrolases. Support for this interpretation comes from the fact that the "unnatural" reactions are somewhat slower than the "natural" ones and the fact that the hydrolase activity toward galactosyl sphingosine is severely deficient in Krabbe's disease (5).

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REFERENCES

1. Hajra, A.K., D.M. Bowen, Y. Kishimoto, and N.S.

Radin, J. Lipid Res. 7:379 (1966).
2. Brady, R.O., J. Kanfer, and D. Shapiro, J. Biol. Chem. 240:39 (1965).
3. Bowen, D.M., and N.S. Radin, J. Neurochem. 16:501 (1969).
4. Dawson, G., J. Lipid Res. 13:207 (1972).
5. Miyatake, T., and K. Suzuki, J. Biol. Chem. 247:5398 (1972).
6. Bowen, D.M., and N.S. Radin, Biochim. Biophys. Acta 152:599 (1968).
7. Brady, R.O., A.E. Gal, J.N. Kanfer, and R.M. Bradley, J. Biol. Chem. 240:3766 (1965).
8. Ho, M.W., J.S. O'Brien, N.S. Radin, and J.S. Erickson, Biochem. J. 131:173 (1973).
9. Suzuki, K., and Y. Suzuki, Proc. Nat. Acad. Sci. 66:302 (1970).
10. Radin, N.S., R.C. Arora, M.D. Ullman, A.L. Brenkert, and J. Austin, Res. Comm. Chem. Pathol. Pharmacol. 3:637 (1972).
11. Brady, R.O., J.N. Kanfer, R.M. Bradley, and D. Shapiro, J. Clin. Invest. 45:1112 (1966).
12. Svennerholm, L., in "Inborn Disorders of Sphingolipid Metabolism," Edited by S.M. Aronson and B.W. Volk, Pergamon Press, Oxford, England, 1967, p. 173.
13. Erickson, J.S., and N.S. Radin, J. Lipid Res. 14:133 (1973).
14. Kopaczyk, K.C., and N.S. Radin, Ibid. 6:140 (1965).
15. Radin, N.S., in "Methods of Enzymology," Vol. 28, Edited by V. Ginsburg, Academic Press, New York, N.Y., 1972, p. 300.
16. Gaver, R.C., and C.C. Sweeley, JAOCS 42:294 (1965).
17. Radin, N.S., and R.C. Arora, J. Lipid Res. 12:256 (1971).
18. Kishimoto, Y., W.E. Davies, and N.S. Radin, Ibid. 6:525 (1965).
19. Dawson, G., A.C. Stoolmiller, and N.S. Radin, Trans. Amer. Soc. Neurochem. 4:92 (1973).
20. Cleland, W.W., and E.P. Kennedy, J. Biol. Chem. 235:45 (1960).
21. Curtino, J.A., and R. Caputto, Lipids 7:525 (1972).

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A New Reaction of Unsaturated Fatty Acid Hydroperoxides: Formation of 11-Hydroxy-12,13-epoxy-9-octadecenoic Acid from 13-Hydroperoxy-9,11-octadecadienoic Acid

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ABSTRACT

One of the main compounds formed from 13L-hydroperoxy-9*cis*,11*trans*-octadecadienoic acid anaerobically at 100 C in aqueous ethanol was found to be *threo*-11-hydroxy-12,13-epoxy-9-octadecenoic acid. The major part (ca. 90%) of this compound was formed from the fatty acid hydroperoxide in a reaction involving *cis*-addition to the Δ^{11} double bond of the proximally linked hydroperoxide oxygen and hydroxyl ion or hydroxyl radical from the solvent. A small part (ca. 10%) was formed by *cis*-addition of the two hydroperoxide oxygens to the Δ^{11} double bond. 11-Hydroxy-12,13-epoxy-9-octadecenoic acid and its isomer, tentatively identified as 11-hydroxy-9,10-epoxy-12-octadecenoic acid, also were isolated from a sample of autoxidized linoleic acid.

INTRODUCTION

Considerable work has been carried out on thermal reactions of unsaturated fatty acids and their oxidation products (1). Surprisingly, little attention has been paid to the thermochemistry of pure, nonisomeric fatty acid hydroperoxides, although such compounds can be prepared easily by lipoxygenase catalyzed oxygenation of naturally occurring polyunsaturated fatty acids (2-5). For example, commercial preparations of soybean lipoxygenase convert linoleic acid mainly into 13L-hydroperoxy-9*cis*,11*trans*-octadecadienoic acid. To gain information about the complex chemistry of thermally reacting fatty acid hydroperoxides, it seemed appropriate to heat a pure fatty acid hydroperoxide, to isolate one of the major compounds from the reaction mixture, to determine its structure, and to elucidate the mechanism of its formation. The present work was carried out along these lines and is concerned with a novel hydroxy-epoxy acid formed from 13L-hydroperoxy-9*cis*,11*trans*-octadecadienoic acid.

MATERIALS

[1-^{14}C] 13L-Hydroperoxy-9*cis*,11*trans*-octadecadienoic Acid

[1-^{14}C] Linoleic acid (New England Nuclear Chemicals, diluted with unlabeled material until 11 μCi/mmole) was incubated with soybean lipoxygenase (Sigma Chemical Co.) at 0 C in sodium borate buffer pH 10.0 (5). The product was subjected to silicic acid chromatography (20 mg material/1 g of Mallinckrodt 100 mesh SiO_2 activated at 120 C). Unreacted linoleic acid was eluted with diethyl ether-petroleum ether (5:95, v/v; 30 ml/1 g SiO_2), and linoleic acid hydroperoxide was eluted with diethyl ether-petroleum ether (15:85, v/v; 30 ml/1 g SiO_2). Analysis of the methyl ester of the hydroperoxide by thin layer radiochromatography indicated a radiochemical purity of 95%. However, there was also a small amount (4-5% of total hydroperoxide) of the isomeric hydroperoxide oxygenated at carbon-9 as shown by thin layer chromatographic (TLC) analysis of the derived saturated hydroxy esters (5).

threo-11,12-Dihydroxy-1-octadecanol

cis-Vaccenic acid (50 mg) (Sigma Chemical Co.) was hydroxylated with 0.5 ml formic acid and 0.1 ml 30% hydrogen peroxide at room temperature for 3 hr (6). Material isolated by extraction with diethyl ether was dissolved in 10 ml dry tetrahydrofurane and refluxed with 100 mg $LiAlH_4$ for 4 hr. Extraction with diethyl ether gave 40 mg (75%) white solid that was homogenous on TLC using sodium arsenite-impregnated plates (R_f=0.57, see below). A single peak appeared on gas liquid chromatographic (GLC) analysis of the trimethylsilyl (TMSi) ether derivative (equivalent chain length, C-22.0; column, 1% SE 30). The mass spectrum showed prominent ions at m/e 503 (M-15; loss of $\cdot CH_3$), 433 (M-85; loss of $\cdot[CH_2]_5CH_3$), 331 (M-187; loss of $\cdot CH[OTMSi]\text{-}[CH_2]_5CH_3$), 289 (M-229; loss of $\cdot[CH_2]_{10}OTMSi$), 261, and 187 ($TMSiO^+$ $=CH\text{-}[CH_2]_5CH_3$) confirming the identity of the parent dihydroxyoctadecanol with 11,12-dihydroxy-1-octadecanol. That the alcohol groups at C-11 and C-12 indeed had the *threo* configuration followed from the method of preparation, *trans*-hydroxylation of a *cis* double bond (6), and from the TLC data (see below).

erythro-11,12-Dihydroxy-1-octadecanol

trans-Vaccenic acid (50 mg) (Sigma Chemical Co.) was hydroxylated with performic acid

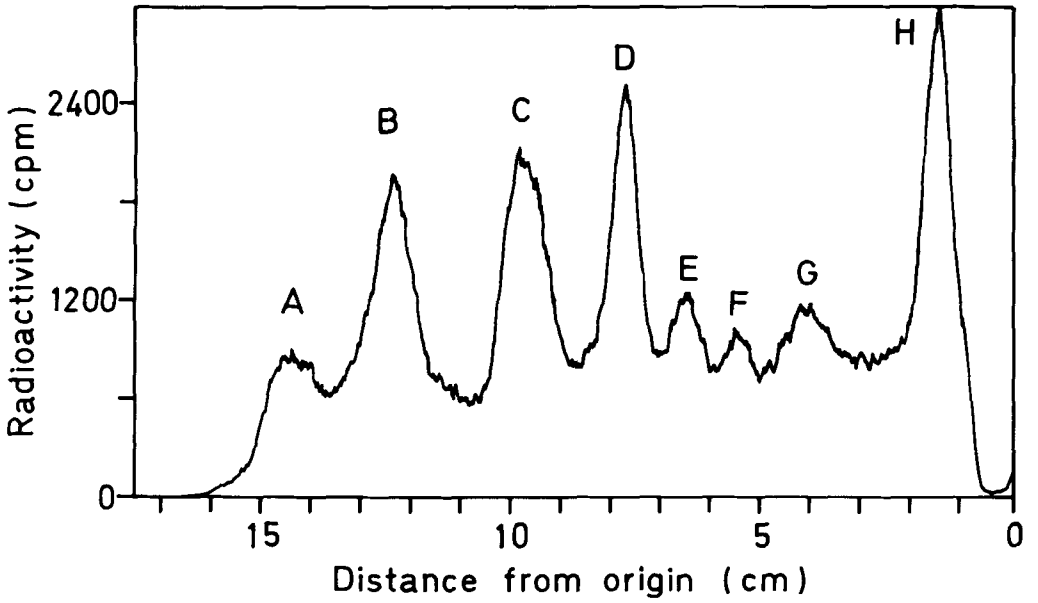

FIG. 1. Thin layer radiochromatogram of esterified material isolated after heat treatment of 4 mg [1-^{14}C] 13L-hydroperoxy-9,11-octadecadienoic acid. Solvent system: organic layer of ethyl acetate-2,2,4-trimethylpentane-water (50:100:100, v/v/v).

and subsequently reduced with $LiAlH_4$, as described above. The mass spectrum of the TMSi derivative (equivalent chain length, C-22.1) was similar to that of the TMSi derivative of the *threo* isomer. A single spot (R_f=0.48) was noted on TLC with sodium arsenite-impregnated plates. That the configurational assignment of the two dihydroxyoctadecanols was correct was supported by the TLC data, since the *threo* isomers of a number of dihydroxystearates previously have been found to move faster than the *erythro* isomers on sodium arsenite-impregnated plates (7).

METHODS

Micro Periodic Acid Oxidation

One μmole of vic-glycol was dissolved in 0.8 ml dry diethyl ether containing 2.4 mg periodic acid (8). The mixture was stirred for 60 min at room temperature. An aliquot (5-10 μl) was injected to an F&M Biomedical gas chromatograph model 402 equipped with a column of 15% silicone grease on Gaschrom Q. Temperature programing (5 C/min) between 90 C and 190 C was used. When methyl *erythro*-9,10-dihydroxystearate was oxidized in this way, two peaks appeared (retention times, 8 and 16 min). The identity of the former peak with nonanal and the latter peak with methyl 9-oxononanoate was established by mass spectrometry.

Chromatographic Methods

Thin layer chromatography of fatty acid hydroperoxides and their reaction products was carried out on plates coated with Silica Gel G. The *erythro* and *threo* forms of 11,12-dihydroxy-1-octadecanol were separated by TLC on plates coated with Silica Gel G-$NaAsO_2$ (9:1, w/w) and methanol-chloroform (3:97, v/v) as solvent. Spots and bands were located by spraying with 2′,7′-dichlorofluorescein and viewing by UV. Radioactivity assay of TLC plates was performed with a Berthold Dünnschichtscanner II.

Gas liquid chromatography was performed with an F&M Biomedical gas chromatograph model 402 and columns of 1% SE 30 on Gaschrom Q (long chain compounds) or 15% Dow Corning silicone grease on Gaschrom Q (short chain compounds). Retention times were expressed as equivalent chain lengths using methyl esters of saturated monocarboxylic acids as standards. Mass spectra were obtained with an LKB 9000 instrument equipped with a column of 1% Dexsil on Chromosorb W (Analabs).

RESULTS

[1-^{14}C] 13L-Hydroperoxy-9*cis*,11*trans*-octadecadienoic acid, 4 mg, was dissolved in 1.6 ml ethanol, and 2.4 ml water was added. The opalescent solution was kept under argon in a sealed vessel at 100 C for 3 hr. After this time the solution was diluted with water, acidified to pH 3 and extracted with three portions of

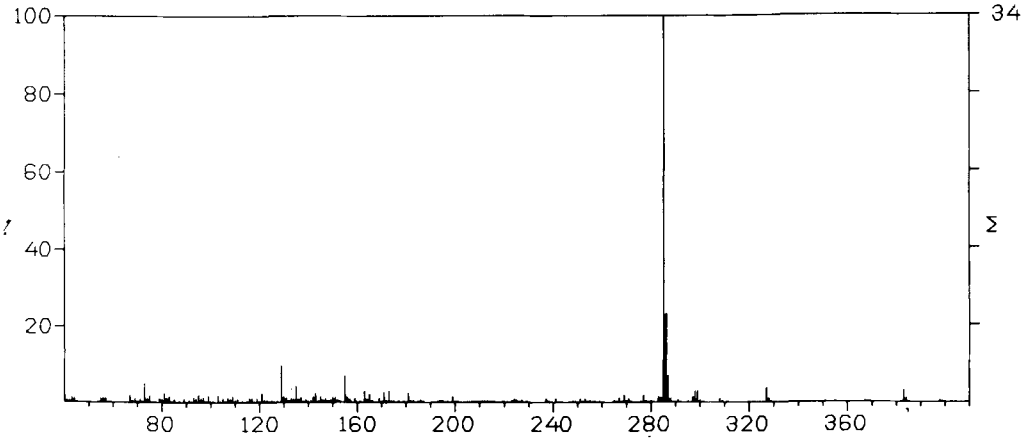

FIG. 2. Mass spectrum of trimethylsilyl ether derivative of compound D.

diethyl ether. The combined ether phases were washed with water until neutral and taken to dryness. The residue, containing ca. 95% initial radioactivity, was treated with ethereal diazomethane and subjected to preparative TLC. At least eight peaks of radioactive material appeared (Fig. 1).

Structure of Compound D

Material in the zone corresponding to peak D (R_f=0.44; 10% of the initial radioactivity) (Fig. 1) was eluted with ether from the silica gel. Analysis of an aliquot by UV spectrometry (Zeiss recording spectrophotometer model DMR 21) showed no absorption band at 234 mμ, indicating that the pair of conjugated double bonds of the starting material was not retained in compound D. Another part was treated with hexamethyldisilazane and trimethylchlorosilane in pyridine and analyzed by GLC. A single peak with a retention time corresponding to C-20.7 appeared (column, 1% SE 30). The mass spectrum is given in Figure 2. The m/e value of the molecular ion (398) suggested a C_{18} fatty acid methyl ester with one double bond, one trimethylsilyloxy group, and one keto or epoxy oxygen. Elimination of a fragment with mol wt 113 gave the base peak at m/e 285. This fragmentation agreed with the partial structure -CH(OTMSi)-CH(O)-CH-$(CH_2)_4CH_3$ which would undergo facile cleavage as indicated. Cleavage on the other side of the epoxy group with elimination of $\cdot(CH_2)_4CH_3$ gave an ion with m/e 327. The presence of an epoxy group in compound D was indicated by IR spectrometry (Perkin Elmer model 257 IR spectrophotometer, chloroform solution). Bands were seen inter alia at the following wavelengths: 2.8-3.0 μ (hydroxyl), 5.78 μ (ester), and 11.2 μ (epoxy) (9). A weak absorption band at 10.3 μ also was seen indicating that the double bond configuration was *trans* in part of the molecules.

The chemical transformations carried out on compound D are summarized in Figure 3. Catalytic hydrogenation of compound D (7 mg) gave a dihydro derivative. The mass spectrum of the TMSi ether showed a molecular ion at m/e 400 and a base peak at m/e 287. The IR spectrum showed bands at 2.8-3.0 μ (hydroxyl), 5.78 μ (ester), and 11.2 μ (epoxy) but no band at 10.3 μ.

The double bond was located by oxidative ozonolysis. Treatment of compound D (0.5 mg) with acetic anhydride-pyridine (1:1, v/v) yielded the acetate derivative (equivalent chain length, 21.1; column, 1% SE 30). Treatment of this material with O_3 in chloroform followed by acetic acid-hydrogen peroxide (5) gave a product that was esterified and subjected to TLC (solvent system, diethyl ether-petroleum ether [3:7, v/v]). A major peak of radioactivity with R_f=0.71 appeared (reference, dimethyl azelate, R_f=0.71). The labeled material was recovered from the silica gel and subjected to GLC (column, 15% silicone grease). A single peak with retention time corresponding to C-12.0 appeared (reference, dimethyl azelate, C-12.0). Mass spectrometry conclusively established the identity of the labeled degradation product as dimethyl azelate.

In another experiment the dihydro derivative of compound D (1 mg) was refluxed with 0.5 ml glacial acetic acid for 2 hr. The residue obtained after evaporation was treated with N NaOH in aqueous methanol at 70 C for 2 hr. Extraction with ethyl acetate yielded a white solid that was converted into the methyl ester and subjected to preparative TLC (solvent

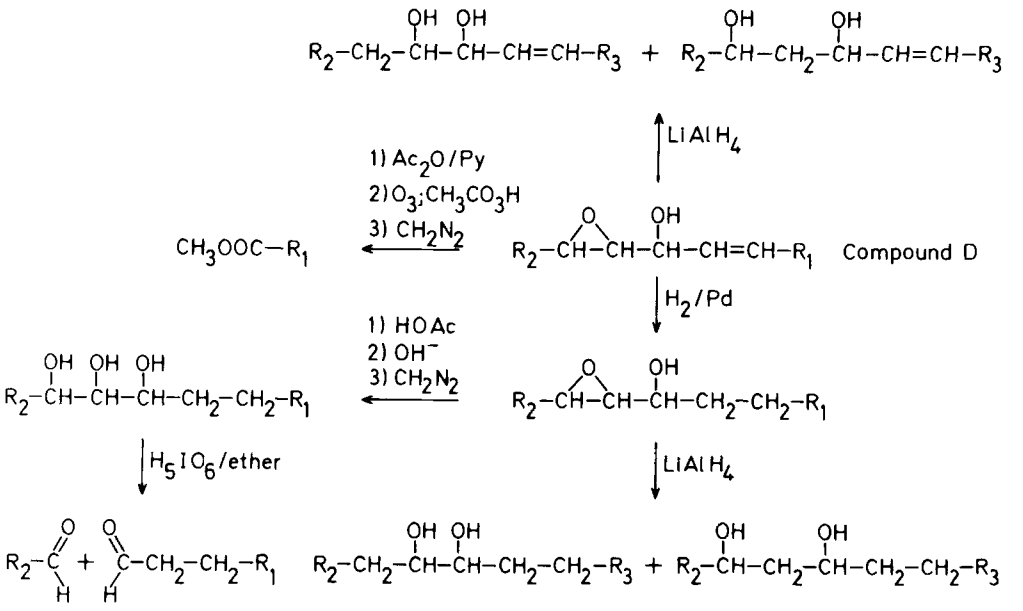

FIG. 3. Reactions used in structure determination of compound D. R_1, $(CH_2)_7COOCH_3$; R_2, $(CH_2)_4CH_3$; R_3, $(CH_2)_7CH_2OH$.

system, organic layer of ethyl acetate-2,2,4-trimethylpentane-water (100:50:100, v/v/v). A major peak of radioactivity appeared (R_f=0.68). This material was recovered and analyzed by GLC as the TMSi derivative. A single peak appeared (C-23.3). The mass spectrum showed ions at m/e 531 (M-31; loss of $\cdot OCH_3$), 462 (M-100; loss of CHO-$[CH_2]_4CH_3$) (10), 389 (M-173; loss of $\cdot CH[OTMSi]$-$[CH_2]_4CH_3$), 287 (M-275; loss of $\cdot CH[OTMSi]$-$CH[OTMSi]$-$[CH_2]_4$-CH_3), and 173 ($TMSiO^+$=CH-$[CH_2]_4CH_3$), thus strongly indicating a C_{18} methyl ester having trimethylsilyloxy groups in positions 11, 12, and 13. Micro periodic acid oxidation performed on the trihydroxy ester yielded hexanal (retention time, 2 min) and methyl 11-oxoundecanoate (retention time, 20 min). The former degradation product gave a mass spectrum identical with that of authentic hexanal. The mass spectrum of the latter component showed ions at m/e 186 (M-28; loss of CH_2=CH_2), 183 (M-31; loss of $\cdot OCH_3$), 171 (M-43; loss of CH_2=CH-O$\cdot$), 139 (M-[43+32]), 87 ($[(CH_2)_2COOCH_3]^+$), and 74 ($[CH_2$=$C(OH)OCH_3]^+$), a fragmentation pattern analogous to that found for methyl 9-oxononanoate prepared by periodic acid oxidation of methyl *erythro*-9,10-dihydroxystearate. These experiments, therefore, showed that the hydrogenated and hydrated derivative of compound D was methyl 11,12,13-trihydroxystearate.

Compound D (0.5 mg) finally was refluxed with 50 mg $LiAlH_4$ in 5 ml dry tetrahydrofurane for 18 hr. The product was converted into the TMSi derivative and subjected to GLC. Two partly separated peaks appeared (C-21.8 and 22.0). The mass spectrum recorded on the first peak showed ions at m/e 501 (M-15; loss of $\cdot CH_3$); 426 (M-90; loss of TMSiOH), 355 (M-[90+71]; loss of TMSiOH plus $\cdot[CH_2]_4CH_3$), 329 (M-187; loss of $\cdot CH_2$-$CH[OTMSi]$-$[CH_2]_4CH_3$), 187 ($[CH_2$-$CH(OTMSi)$-$(CH_2)_4$-$CH_3]^+$), and 173 ($TMSiO^+$=CH-$[CH_2]_4CH_3$) (TMSi derivative of 11,13-dihydroxy-1-octadec-9-enol), whereas the mass spectrum of the latter peak showed ions at m/e 501 (M-15; loss of $\cdot CH_3$), 329 (M-187; loss of $\cdot CH[OTMSi]$-$[CH_2]_5CH_3$), and 187 ($TMSiO^+$=CH-$[CH_2]_5CH_3$) (TMSi derivative of 11,12-dihydroxy-1-octadec-9-enol).

Configuration at Carbons 11 and 12

To establish whether the configuration of the oxygen bearing carbon atoms 11 and 12 was *erythro* or *threo* the following experiment was carried out (Fig. 3). The dihydro derivative of compound D (5 mg) was reduced with $LiAlH_4$, as described above; and the product was subjected to TLC using sodium arsenite-impregnated plates. Two peaks of radioactivity appeared (ratio 1:1; R_f=0.57 and R_f=0.48). The references, *threo*-11,12-dihydroxy-1-octadecanol and *erythro*-11,12-dihydroxy-1-octadecanol were also chromatographed at R_f=0.57 and 0.48, respectively. The zones containing

TABLE I

Isotope Composition of Methyl 11-Hydroxy-12,13-epoxy-9-octadecenoate Formed in the Presence of $H_2{}^{18}O$ and from $^{18}O_2$-Labeled 13L-Hydroperoxy-9,11-octadecadienoic Acid

Experiment	Derivative analyzed	M^+-15	Isotopic composition of ions		
			I[a]	II[b]	III[c]
$H_2{}^{18}O$	Compound D-TMSi	52% $^{18}O_1$ 48% $^{18}O_0$	52% $^{18}O_1$ 48% $^{18}O_0$	---	---
$H_2{}^{18}O$	Compound D-H_2-TMSi	52% $^{18}O_1$ 48% $^{18}O_0$	---	52% $^{18}O_1$ 48% $^{18}O_0$	50% $^{18}O_1$ 50% $^{18}O_0$
$^{18}O_2$	Compound D-TMSi	8% $^{18}O_2$ 71% $^{18}O_1$ 21% $^{18}O_0$	0% $^{18}O_2$ 8% $^{18}O_1$ 92% $^{18}O_0$	---	---

[a] I = CH_3OOC-$(CH_2)_7$-CH=CH-CH=O^+TMSi.
[b] II = CH_3OOC-$(CH_2)_9$-CH=O^+TMSi.
[c] III = $TMSiO^+$=CH-CH$\overset{}{\underset{O}{—}}$CH-$(CH_2)_4CH_3$.

the labeled material were scraped off and added to 2N NaOH in aqueous methanol to hydrolyze the arsenite complexes (6). After 18 hr at room temperature, the solution was extracted with diethyl ether, and the material obtained converted into the TMSi derivative and analyzed by GLC-mass spectrometry. The mass spectrum of the TMSi derivative of the faster moving compound (R_f=0.57) showed ions at m/e 503, 433, 331, 289, 261, and 187 (identical with the mass spectrum of TMSi derivative of *threo*-11,12-dihydroxy-1-octadecanol, see above). The mass spectrum of the slower moving compound (R_f=0.48) showed prominent ions at m/e 503 (M-15; loss of $\cdot CH_3$), 428 (M-90; loss of TMSiOH), 357 (M-[90+71]; loss of TMSiOH plus $\cdot[CH_2]_4$-CH_3), 331 (M-187; loss of $\cdot CH_2$-CH[OTMSi]-$[CH_2]_4CH_3$), and 173 ($TMSiO^+$=CH-$[CH_2]_4CH_3$) (TMSi derivative of 11,13-dihydroxy-1-octadecanol). The cross contamination of the two isomeric dihydroxyoctadecanols was 5% or less. Consequently, the 11,12-dihydroxy-1-octadecanol formed from compound D had the *threo* configuration.

Experiments with ^{18}O-Enriched Water

13L-Hydroperoxy-9,11-octadecadienoic acid (1 mg) was dissolved in 0.32 ml ethanol, and 0.48 ml ^{18}O-enriched water (60% ^{18}O, 40% ^{16}O; Miles Laboratories) was added. The solution was kept at 100 C under argon for 3 hr, and compound D was isolated, as described above. Mass spectrometric analysis was made on the TMSi ethers of compound D and of the dihydro derivative of compound D (Table I). As seen from the ion formed by elimination of $\cdot CH_3$, compound D formed in the presence of $H_2{}^{18}O$ was a mixture of 52% molecules containing one atom of ^{18}O and 48% molecules containing no ^{18}O. Since the ions formed by elimination of $\cdot CH\overset{}{\underset{O}{—}}CH$-$(CH_2)_4CH_3$ (I and II), also were mixtures of 52% containing one ^{18}O and 48% without ^{18}O, the heavy oxygen must be located at C-11. The ion III, formed by elimination of the carboxyl chain, had ca. the same isotopic composition, showing that there was little, or no, incorporation of ^{18}O into the carboxyl group during the heat treatment. Since the ^{18}O-enriched water was a mixture of 60% $H_2{}^{18}O$ and 40% $H_2{}^{16}O$, it was apparent that the major part (ca. 87%) of 13-hydroperoxy-9,11-octadecadienoic acid incorporated one oxygen, and a minor part (ca. 13%) did not incorporate oxygen from the solvent during its conversion into compound D.

Experiments with $^{18}O_2$-Labeled 13L-Hydroperoxy-9,11-octadecadienoic Acid

$^{18}O_2$-Labeled 13L-hydroperoxy-9,11-octadecadienoic acid was prepared by incubation of linoleic acid (120 mg) with soybean lipoxygenase under $^{18}O_2$ atmosphere (11) ($^{18}O_2$, 98%, Miles Laboratories). An aliquot of the linoleic acid hydroperoxide thus obtained was treated with $SnCl_2$ in ethanol (5) to prepare $^{18}O_1$-labeled 13L-hydroxy-9,11-octadecadienoic acid. Mass spectrometric analysis of the TMSi derivative demonstrated the presence of 79% molecules with one ^{18}O and 21% molecules without ^{18}O. That the ^{18}O was located at C-13 also was apparent from the mass spectrum (ion at m/e 292 due to elimination of $TMSi^{18}OH$). Since there should be a negligible amount of the species ^{16}O-^{18}O present during the incubation, the isotopic composition of the hydroperoxide should be the same as that of the hydroxy acid, i.e. 79% $^{18}O_2$ molecules and 21% molecules without ^{18}O. Table I shows the isotopic composition of compound D formed from the $^{18}O_2$-labeled hydroperoxide. From

the ion formed by elimination of $\cdot CH_3$, it may be seen that the $^{18}O_2$-hydroperoxide yielded predominantly molecules of compound D containing one atom of ^{18}O (71/79x100%=90%) and, to a small extent, molecules containing two atoms of ^{18}O (8/79x100%=10%). The former molecules contained ^{18}O at carbons 12 and 13 (epoxy oxygen), as shown by the loss of ^{18}O in the elimination of the epoxy group-containing fragment. The latter molecules contained ^{18}O in the epoxy group, as well as in the hydroxyl group attached to carbon 11.

Isolation of Compound D and its Isomer from Autoxidized Linoleic Acid

Linoleic acid, 100 mg, was refluxed under air in 50 ml 40% aqueous ethanol for 22 hr. The solution was acidified and extracted twice with diethyl ether. Aliquots of the esterified residue were subjected to TLC (solvent system: organic layer of ethyl acetate-2,2,4-trimethylpentane-water [50:100:100, v/v/v]). In addition to an intense band at the solvent front corresponding to methyl linoleate, a large number of bands due to more polar derivatives appeared. One band of medium intensity had the same R_f as that of compound D (R_f=0.44). This material was converted into the TMSi derivative and analyzed by GLC mass spectrometry. A major peak appeared (C-20.7). Several mass spectra recorded on different parts of the peak indicated the presence of two isomers, both having the molecular ion at m/e 398. The mass spectra recorded on the ascending part of the peak showed ions inter alia at m/e 398 (M^+), 383 (M-15), 327 (M-71; loss of $\cdot C_5H_{11}$), and 285 (M-113; loss of $\cdot CH\overset{}{\underset{O}{—}}CH-[CH_2]_4CH_3$), whereas the mass spectra recorded on the descending part showed ions inter alia at m/e 398 (M^+), 383 (M-15), 241 (M-157; elimination of $\cdot[CH_2]_7-COOCH_3$), and 199 ($TMSiO^+=CH-CH=CH-[CH_2]_4CH_3$). The more rapidly eluting isomer could be identified as the TMSi derivative of methyl 11-hydroxy-12,13-epoxy-9-octadecenoate (compound D) by comparing the mass spectra with that of the authentic derivative. The more slowly eluting isomer tentatively was identified as the TMSi derivative of methyl 11-hydroxy-9,10-epoxy-12-octadecenoate on basis of the mass spectrometric fragmentation. As judged from the intensities of the base peaks at m/e 285 and m/e 199 ca. equal amounts of the two isomers were present.

DISCUSSION

At least eight compounds were present in the product isolated after heat treatment under anaerobical conditions of 13L-hydroperoxy-9,11-octadecadienoic acid (Fig. 1). Compound D, one of the major components, was isolated and found to be *threo*-11-hydroxy-12,13-epoxy-9-octadecenoic acid. The location of the functional groups was established mainly by mass spectrometric analysis of derivatives of compound D and by chemical degradation. A micro method for periodic acid oxidation in ether combined with direct analysis of the products by GLC-mass spectrometry was developed in the course of this work. This method proved convenient in determining the position of vic-glycol groups in dihydroxystearates and the position of the three alcohol groups in the hydrated and hydrogenated derivative of compound D.

A series of derivatives related to compound D, e.g. methyl 11-(2,2,5,7,8-pentamethyl-6-oxychroman)-*cis*-12,13-epoxy-9*trans*-octadecenoate, recently were shown to be formed by radical addition of linoleic acid hydroperoxides to α-tocopherol or an analogous hydroxychroman in the presence of Fe^{3+} or proflavin sensitized by visible light (12). In contrast there was no need for metal ions or irradiation in the formation of compound D of the present study. Another related compound, 9-hydroxy-*cis*-12,13-epoxy-10*trans*-octadecenoic acid, has been isolated after incubation of linoleic acid with wheat flour extracts (13). Notably, this epoxide apparently was not formed from linoleic acid hydroperoxide but from an intermediate formed from linoleic acid and wheat flour lipoxygenase (13).

To assess the relative configuration of carbons 11 and 12, compound D was hydrogenated and reduced with lithium aluminium hydride (Fig. 3). As expected this treatment gave two isomeric dihydroxyoctadecanols, i.e. 11,12-dihydroxy- and 11,13-dihydroxy-1-octadecanols, which were separated by TLC on sodium arsenite-impregnated plates. By comparison with authentic *erythro*- and *threo*-11,12-dihydroxy-1-octadecanols, it could be demonstrated that the 11,12-dihydroxyoctadecanol derived from compound D had the *threo* configuration. Since the two carbon-oxygen bonds at C-11 and C-12 of compound D are not involved in the conversion into 11,12-dihydroxy-1-octadecanol, it followed that compound D had the *threo* configuration at carbons 11 and 12. Since the Δ^{11} double bond of the parent fatty acid hydroperoxide had the *trans* configuration the two oxygens at carbons 11 and 12,13 of compound D must have been incorporated by *cis* addition, i.e. addition on the same side of the plane of the Δ^{11} double bond.

The origin of the oxygen atoms at C-11 and

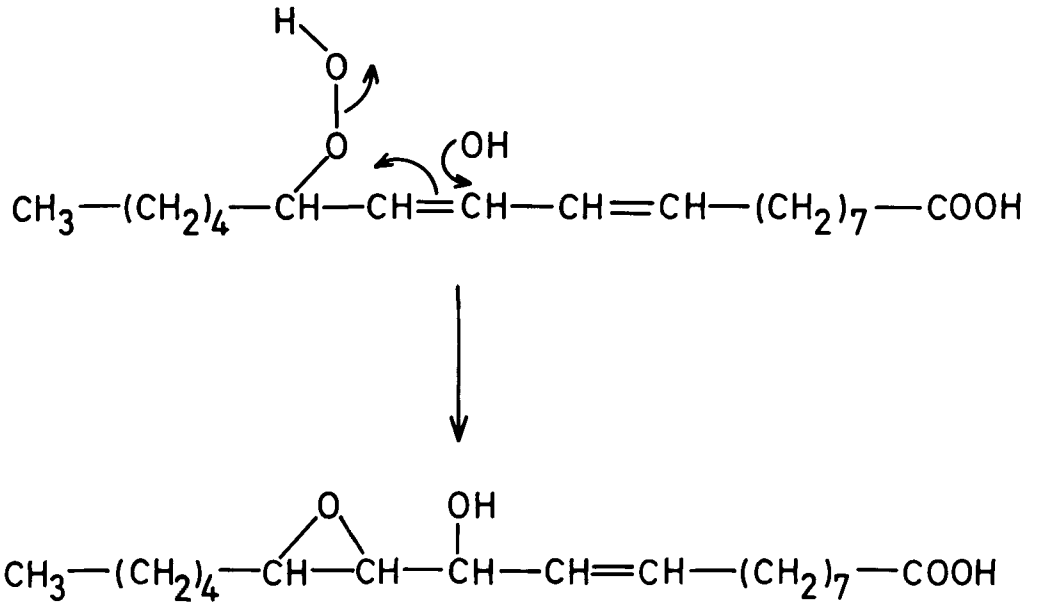

FIG. 4. Mechanism of formation of 11-hydroxy-12,13-epoxy-9-octadecenoic acid from 13L-hydroperoxy-9,11-octadecadienoic acid.

C-12,13 of compound D was established in experiments with ^{18}O-enriched water and with $^{18}O_2$-labeled 13L-hydroperoxy-9,11-octadecadienoic acid. Heat treatment of the fatty acid hydroperoxide in the presence of $H_2{}^{18}O$ gave rise to compound D labeled with ^{18}O in the hydroxyl group. However, the percentage of ^{18}O-containing molecules (52%) was smaller than the percentage of $H_2{}^{18}O$ in the ^{18}O-enriched water (60%) showing that a small part (13%) of compound D was formed without incorporation of OH from the solvent. Compound D isolated after heat treatment of 13L-hydroperoxy-9,11-octadecadienoic acid labeled with $^{18}O_2$ in the hydroperoxy group largely retained one atom of ^{18}O. This atom was present in the epoxy group. A small part (10%) retained two atoms of ^{18}O, located in the hydroxy and epoxy groups.

The mechanism proposed for the reaction, by which the major part of the hydroxy-epoxy acid was formed, involves elimination of OH from the hydroperoxy group and *cis*-addition to the Δ^{11} double bond of the proximally linked hydroperoxide oxygen and OH from the solvent (Fig. 4). The nature of OH (OH^- or $\cdot OH$), i.e. whether or not the reaction involves free radicals, has not been established conclusively. The structure of compound B (Fig. 1), tentatively identified as methyl 11-ethoxy-12,13-epoxy-9-octadecenoate, gave additional support for this mechanism in which an ion or radical from the solvent (aqueous ethanol) was bound to carbon 11.

There seems to be two possible mechanisms for the minor reaction by which the hydroxy-epoxy acid was formed without incorporation of oxygen from the solvent. One consists of dissociation of OH from the hydroperoxide group, as described above, followed by rebinding to carbon 11 before equilibration with solvent molecules. Alternatively there is a *cis*-addition of the two hydroperoxide oxygens to the Δ^{11} double bond without prior cleavage of the O-O bond.

The formation of 11-hydroxy-12,13-epoxy-9-octadecenoic acid and its isomer, 11-hydroxy-9,10-epoxy-12-octadecenoic acid, during autoxidation of linoleic acid probably occurs by way of the two initial products of linoleate autoxidation, i.e. 13-hydroperoxy-9,11-octadecadienoic acid and 9-hydroperoxy-10,12-octadecadienoic acid. Further studies on the formation of hydroxy-epoxy acids as well as polyhydroxylated acids during autoxidation of linoleic acid are in progress and will be reported later.

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REFERENCES

1. Figge, K., Chem. Phys. Lipids 6:1591 (1971).
2. Hamberg, M., and B. Samuelsson, Biochem. Biophys. Res. Commun. 21:531 (1965).
3. Dolev, A., W.K. Rohwedder, and H.J. Dutton, Lipids 2:28 (1967).

4. Hamberg, M., and B. Samuelsson, J. Biol. Chem. 242:5329 (1967).
5. Hamberg, M., Anal. Biochem. 43:515 (1971).
6. Morris, L.J., and D.M. Wharry, Lipids 1:41 (1966).
7. Morris, L.J., in "New Biochemical Separations," Edited by A.T. James and L.J. Morris, van Nostrand Co., Ltd., London, England, 1964, p. 295.
8. Ireland, R.E., and J. Newbould, J. Org. Chem. 28:23 (1963).
9. Shreve, O.D., M.R. Heether, H.B. Knight, and D. Swern, Anal. Chem. 23:277 (1951).
10. Capella, P., and C.M. Zorzut, Ibid. 40:1458 (1968).
11. Dolev, A., W.K. Rohwedder, T.L. Mounts, and H.J. Dutton, Lipids 2:33 (1967).
12. Gardner, H.W., K. Eskins, G.W. Grams, and G.E. Inglett, Ibid. 7:324 (1972).
13. Graveland, A., JAOCS 47:352 (1970).

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Fungi Pathogenic to Insects: III. Neutral and Polar Lipids of *Entomophthora coronata*[1]

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ABSTRACT

The neutral and polar lipid composition of *Entomophthora coronata* was determined qualitatively. The fungus was grown on a chemically nondefined medium (Sabouraud dextrose yeast extract) and a chemically defined medium for a period up to 26 days. The lipids were characterized by thin-layer, column, gas chromatography, and selective sprays, ^{32}P-labeling, and mass spectrometry. The neutral lipids consist of monoglycerides, diglycerides, cholesterol, free fatty acids, triglycerides, and cholesteryl esters. The polar lipids consist of phospholipids (phosphatidyl ethanolamine, phosphatidyl choline, lysophosphatidyl ethanolamine, lysophosphatidyl choline, and spingomyelin), a number of glycolipids including cerebrosides, and many unrecognizable lipids, most of which are present in trace amounts. The cerebrosides and spingomyelin are present in significant amounts, and their concentration increased with age of the culture. The major fatty acids (>10%) of the total, neutral, and polar lipids of the mycelia are 14:0, 16:0, 18:1, 18:3(γ), and 24:1. The polar lipids of total culture (unsaturation index 0.88) and of the conidia (unsaturation index 1.48) are considerably more unsaturated than the corresponding neutral lipids (unsaturated index 0.50 and 0.49). The mycelial polar lipids, compared to the neutral lipids, possess less 14:0 and 18:1 but contain a greater percentage of 16:0, 18:2, 18:3(γ), 24:0, and 24:1. The major fatty acid of the conidia (>10%) are 13:0, 14:0, 18:1, 18:2, 18:3(γ), and 20:4. Their polar lipids have a higher proportion of 18:2, 18:3(γ), and 20:4. The cerebrosides possess 24.1 in high relative proportion (30.1%).

INTRODUCTION

The fatty acid composition of entomogenous fungi has been examined and found to be of some use in indicating taxonomic relationships (1,2). Mumma and Bruszewski have shown that the fungus *Entomophthora coronata* contains several unusual fatty acids (3). Entomogenous fungi are being considered for possible insect control, and they must be studied thoroughly before they can be disseminated in man's environment. There is a lack of knowledge concerning the lipid composition of fungi, particularly fungi pathogenic to animals, when compared to our knowledge concerning the lipid composition of other microorganisms. *E. coronata* has been reported to infect vertebrates on rare occasions (4). In an effort to better understand the lipid characteristics of fungi pathogenic to animals and to characterize chemically the entomogenous fungi, we now report qualitatively the neutral and polar lipid composition of *E. coronata* (5).

EXPERIMENTAL PROCEDURE

Culture of organism: Stock cultures of *E. coronata* (American Type Culture Collection no. 1005) were grown in petri dishes on Sabouraud dextrose yeast extract agar (Difco) for 10 days (3). Erlenmeyer flasks (250 ml) containing 100 ml sterile liquid medium were inoculated with mycelia and conidia using a sterile wire loop. The fungus was grown on two media: a chemically defined medium and a chemically nondefined medium (Sabouraud dextrose yeast extract). The chemically defined medium consisted of 20 g glucose, 2 g L-asparagine, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.6 g KH_2PO_4, and 2.4 g K_2HPO_4/liter. The chemically nondefined medium contained 10 g Neopeptone (Difco), 40 g glucose, and 2 g yeast extract/liter. The organism was grown in stationary culture at 25 C for up to 26 days under constant light. Mycelia were harvested at various times and the lipid extracted, as previously reported (3). In the radiochemical studies culturees were grown on a medium containing 50 μCi of $^{32}PO_4^{-3}$ for 10 days.

Mycelia lipids: The fungal lipids were fractionated into two classes on a 1 x 8 cm silicic acid column using 12 ml chloroform to elute neutral lipids and 12 ml methanol followed by 12 ml chloroform-methanol-water (65:25:4, v/v/v) to elute the polar lipids. The latter two fractions were combined and solvents evaporated under a stream of N_2.

The neutral lipids were fractionated further

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

TABLE I

Percentage Neutral and Polar Lipids of *Entomophthona coronata*

Sample lipids	Day				
Total culture	Sabouraud			Defined	
	10	16	26	16	26
Neutral	86.6	93.2	92.1	87.9	91.8
Polar	13.4	6.8	7.9	12.1	8.2
Conidia	Sabouraud – 7 Day				
Neutral		93.5			
Polar		6.5			

by thin layer chromatography (TLC) (Supelcosil 12A, Supelco, Inc., Belle Fonte, Pa.) employing petroleum ether (30-60 C)-ethyl ether-acetic acid (50:50:1, v/v/v/). Lipids were visualized by charring with 20% H_2SO_4, with selective sprays, or radioautography.

A portion (312 mg) of the neutral lipids was saponified in 0.5 M KOH in 10% aqueous methanol (7 ml) for 4 hr at 45 C with shaking. The unsaponifiables were extracted twice with ethyl ether and the ether washes combined. The ether solvent was evaporated under N_2, and the residue again was dissolved in 1.5 ml ethyl ether. The unsaponifiable lipids were streaked on preparative thin layer chromatographic plates and developed in ethyl ether-benzene-ethanol-acetic acid (40:50:2:0.2, by vol.) (6). The steroid band was located with Rhodamine 6G (0.5% in ethanol), scraped from the plate, placed in a glass wool plugged pasteur pipet and eluted from the absorbent with 3 ml chloroform. The solvent was evaporated under N_2 and the residue dissolved in methanol and cooled overnight. The solution was filtered, and a small amount of crystalline material was obtained.

A portion of the crystalline steroid, as well as the crude unsaponifiable fraction dissolved in ethyl ether was analyzed by gas liquid chromatography (GLC) (3% SE-30 6 ft column at 225 C, Aeorgraph Model 200 gas chromatograph, flame ionization detector, and He carrier gas). A standard mixture of cholestane and cholesterol was used as a reference. The mass spectrum of the isolated sterol was obtained with a Nuclide Model Graf III mass spectrometer utilizing the solid inlet probe.

The polar lipid fraction was spotted on thin-layer chromatographic plates (Supelcosil 12A, Supelco, Inc.) and developed two dimensionally: first direction, chloroform-methanol-water-28% ammonium hydroxide (130:70:8:0.5, v/v/v/v) and the second direction, chloroform-acetone-methanol-acetic acid-water (100:40:20:20:10, by vol.) (7). The identity of some of these lipids was established by the use of selective sprays.

The phospholipids were characterized fur-

TABLE II

Per cent Fatty Acids in Lipids of *Entomophthona coronata*

	26 Day culture				Conidia		
Fatty acid	Total	Neutral	Polar	Cerebrosides	Total	Neutral	Polar
12:0	7.3	7.8	2.2	3.5	5.1	5.2	1.6
13:0	5.4	5.5	0.4	3.0	10.6	11.1	3.8
14:0	39.4	36.1	13.5	16.4	34.9	35.9	18.6
15:0	2.1	3.2	1.9	0.6	3.9	3.3	3.7
16:0	9.7	10.8	20.7	14.1	9.2	9.5	7.2
16:1	0.3	1.1	---	Trace	0.9	0.6	0.4
17:0	0.1	0.9	2.3	Trace	1.2	1.0	0.3
16:2	0.2	0.8	2.3	Trace	0.8	0.6	0.6
18:0	1.9	2.3	7.6	0.5	2.2	2.4	0.8
18:1	21.3	21.3	9.5	14.0	16.5	17.2	10.7
$18:2_{(9,12)}$	3.5	2.9	8.1	2.9	8.7	7.7	31.0
$18:3_{(6,9,12)}$	1.1	0.4	10.2	1.9	1.3	0.9	6.7
18:2	0.4	1.0	Trace	Trace	0.1	0.2	Trace
18:2	4.8	2.5	8.4	0.2	0.2	0.2	Trace
18:3	0.3	Trace	Trace	5.1	Trace	Trace	Trace
$20:3_{(8,11,14)}$	1.1	0.7	Trace	0.6	0.6	0.6	2.7
$20:4_{(5,8,11,14)}$	2.9	2.2	Trace	0.4	2.5	2.4	11.3
20:4	1.3	Trace	Trace	6.6[a]	Trace	Trace	Trace
24:0	Trace	Trace	3.8	Trace	Trace	Trace	Trace
24:1	1.2	0.7	10.4	30.1	1.4	1.3	0.7
% Unsaturated	38.4	33.6	48.9	61.8	33.0	31.7	64.1
USI[b]	0.65	0.50	0.88	1.01	0.54	0.49	1.48

[a]A saturated polar fatty acid.

[b]Average double bonds/mole fatty acid. USI = unsaturated index.

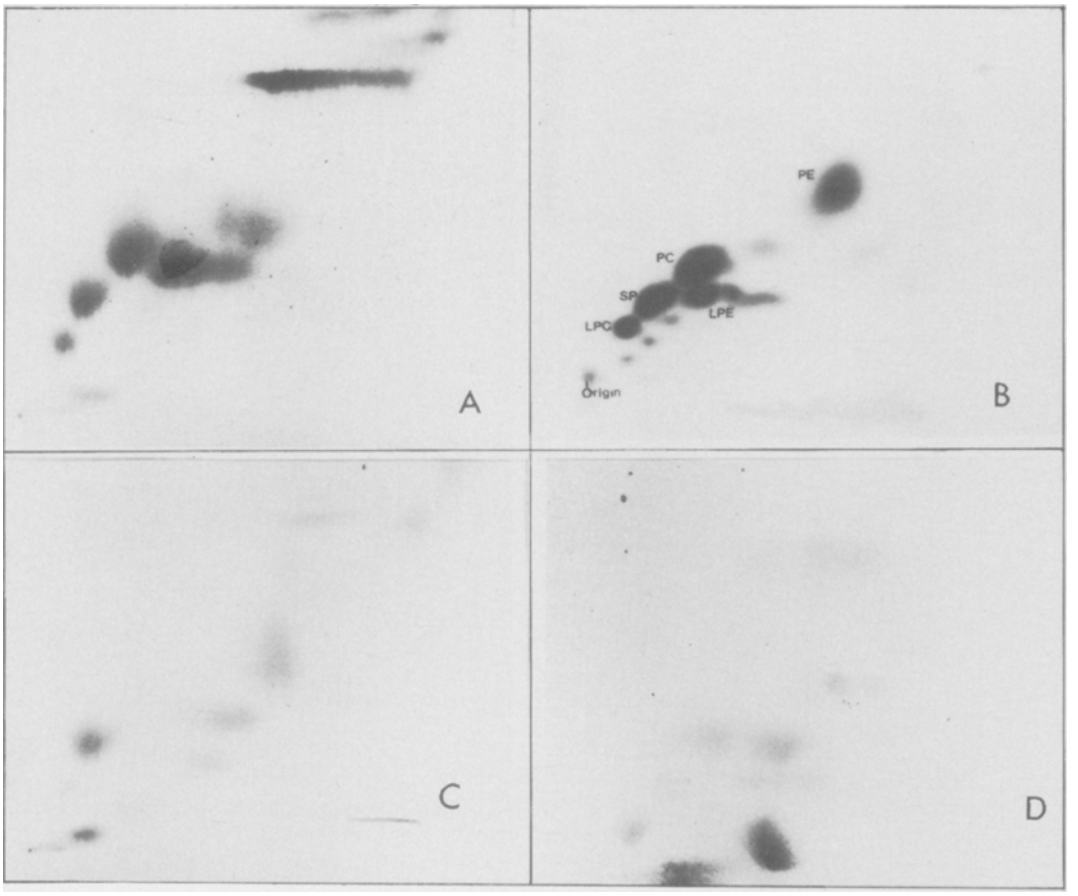

FIG. 1. Thin-layer chromatograms of *Entomophthora coronata* lipids: (1A.) 20% sulfuric acid char of total polar lipids of 10 day old culture; (1B.) radioautogram of ^{32}P-labeled lipids of 10 day old culture; (1C.) 20% sulfuric acid char of total polar lipids of 26 day old culture; and (1D.) α-naphthol sprayed chromatogram of conidia polar lipids. Chromatograms were developed in the first direction (horizontally) with chloroform-methanol-water-28% ammonium hydroxide (130:70:8:0.5, v/v/v/v) and in the second direction with chloroform-acetone-methanol-acetic acid-water (100:40:20:20:10, v/v/v/v/v) (7).

ther by ^{32}P-labeling. The fungus was grown on the chemically defined medium spiked with 50 μCi $^{32}PO_4^{-3}$ for 10 days, and the lipids were extracted and fractionated, as previously mentioned. The individual ^{32}P-labeled lipids were removed from two-dimensional thin-layer chromatographic plates and deacylated by the method of Dawson (8). The deacylated products were assayed by two-dimensional paper chromatography on Whatman no. 4 filter paper. Chromatograms were developed with phenol-water (100:40, w/w) in the first direction and with 1-butanol-propionic acid-water (100:50:70, v/v/v) in the second. Identity of these products was established by comparison of R_fs with those of deacylated ^{32}P-labeled plant lipids (9).

Glycolipids were separated from the other lipids by silicic acid column chromatograpy (10). A portion of the fungal lipids (100-200 mg) was placed on a 1 x 6 cm silicic acid column in a minimal amount of chloroform. A step-wise elution using the following sequence and volumes of solvents was employed: (a) chloroform, 10 column volumes (neutral lipids), (b) acetone, 40 column volumes (glycolipids), (c) methanol, 10 column volumes (phospholipids).

The acetone fraction containing the glycolipids was analyzed by one-dimensional TLC (chloroform-methanol-water, 65:25:4, v/v/v). A standard mixture containing bovine cerebrosides and sulfolipids was used as a reference (Supelco, Inc.). Plates were visualized with an α-naphthol spray (11) and 20% H_2SO_4 char. Trimethylsilyl derivatives of the glycosides and sphingosine bases were prepared and analyzed by GLC, according to the method of Vance and Sweely (12). A 6 ft 15% DEGS column operated at 180 C was used for analysis of the

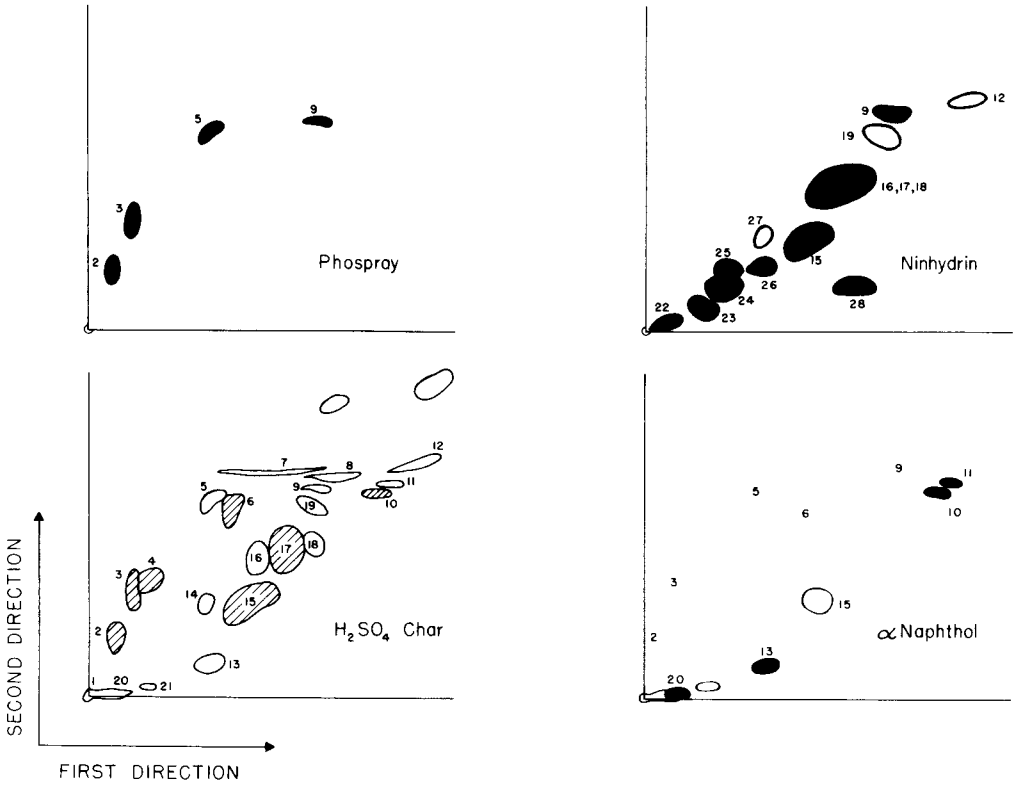

FIG. 2. Results of four separate sprays with thin-layer chromatograms of polar lipids of 14 day old cultures of *Entomophthora coronata*. The solvent systems used were the same as in Figure 1.

methyl esters of the fatty acids. The trimethylsilyl derivatives were analyzed on a 6 ft 2% OV-1 column operated at 160 C until the trimethylsilyl mannitol marker appeared and then the column was temperature programed from 160-200 C at 10 C/min to elute the trimethylsilyl sphingosine bases.

Conidia lipids: *E. coronata* conidia were harvested and extracted as follows: five large petri dishes (150 mm) of Sabouraud-dextrose-yeast agar were inoculated with mycelia. Conidia are ejected forcibly to the petri dish cover where they stick. After 7 days the petri dish covers containing the condida were removed and rinsed with 10 ml chloroform-methanol (2:1). This conidial suspension was transferred to a 50 ml centrifuge tube and allowed to stand for 24 hr at 4 C. The mixture then was centrifuged, the supernatant liquid removed and evaporated under N_2, resulting in 329.2 mg crude lipid. The residue was dissolved in chloroform-methanol (2:1) and Folch washed (13). The lipid layer was evaporated to dryness (253.6 mg), and an aliquot of the residue (133 mg) was dissolved in a minimum amount of chloroform and separated on a siliicic acid column, as previously described (113.8 mg neutral lipids and 7.8 mg polar lipids). The dried extracted conidia weighed 337.1 mg.

RESULTS AND DISCUSSION

The percentages of neutral and polar lipids of *E. coronata* grown on defined and Sabouraud (nondefined) media for 10, 16, and 26 days are presented in Table I. In addition the percentages of neutral and polar lipids in the conidia of *E. coronata* collected on the seventh day of growth also are given in Table I. The percentage of polar lipids in the total culture varied between 6.8 and 13.4% and was greatest in the shorter sampling time. Since the conidia are ejected forcibly and stick to the petri dish cover, they are collected easily, and their lipid extracts are free of any mycelial contamination. However, the total culture extracts always are contaminated with conidial lipids. The conidia possessed 38.4% lipids, of which only 6.5% were polar lipids.

The fatty acid composition of total, neutral, and polar lipids of 26 day old *E. coronata* and of conidia collected on the seventh day are presented in Table II. Although considerable

TABLE III

Reaction of Polar Lipids to Various Spray Reagents

Spot	Spray[a] A	B	C	D	E	F	G	Identification
1	–	–	–	–	+	+	–	
2	+	–	+	–	+	+	+	Lysophosphatidyl choline
3	+	–	–	–	+	+	+	Sphingomyelin
4	–	–	–	–	+	+	+	
5	+	–	+	–	+	+	+	Phosphatidyl choline
6	+	–	–	–	+	+	+	Lysophosphatidyl ethanolamine
7	–	–	–	–	–	+	–	
8	–	–	–	–	+	+	+	
9	+	+	–	–	+	+	–	Phosphatidyl ethanolamine
10	–	–	–	+	+	+	+	Cerebrosides
11	–	–	–	+	+	+	+	Cerebrosides
12	–	+	–	–	+	+	–	
13	–	–	–	+	+	+	+	
14	–	–	–	–	+	+	+	
15	–	–	–	+	+	+	+	
16	–	+	–	–	–	+	–	
17	–	+	–	–	+	+	–	
18	–	+	–	–	–	+	–	
19	–	+	–	–	–	+	–	
20	–	–	–	+	+	+	–	
21	–	–	–	+	–	+	–	
22	–	+	–	–	–	–	–	
23	–	+	–	–	+	–	–	
24	–	+	–	–	–	–	–	
25	–	+	–	–	–	–	–	
26	–	+	–	–	–	–	–	
27	–	+	–	–	–	–	+	
28	–	+	–	–	–	–	–	

[a]A = phospray, Supelco Inc. (14), B = ninhydrin, Stahl #178 (15), C = dragendorf, Stahl #95 (15), D = α-Naphthol (11), E = iodine vapor Stahl #141 (15), F = 20% sulfuric acid char, and G = Schiff's (16).

catabolism has occurred in 26 day old cultures, no appreciable change was observed previously in the total fatty acid composition of *E. coronata* over the entire 26 day growth period (3). The major fatty acids (>10%) are 14:0, 16:0, 18:1, 18:3(γ), and 24:1. The polar lipids of the total culture (unsaturated index [USI] 0.88) and of the conidia (USI 1.48) are considerably more unsaturated than the corresponding neutral lipids (USI 0.50 and 0.49). The mycelial polar lipids, compared to the neutral lipids, possess less 14:0 and 18:1 but contain a greater percentage of 16:0, 18:2, 18:3 (γ), 24.0, and 24:1. The high percentage of 24:0 and 24:1 suggest the presence of glycolipids. The polar conidial lipids contain a high percentage of 18:2, 18:3, and 20:4 which contribute significantly to the overall unsaturation (USI 1.48). The relative concentration of 20:4 is unusually high (11.3%) and raises some question as to what function it may possess in these condidal membrane lipids.

The neutral and polar lipids were fractionated further by TLC. The neutral lipids consisted of triglycerides, steroid esters, free fatty acids, steroids, 1,2-diglycerides, 1,3-diglycerides, and monoglycerides. The steroids were isolated by TLC from the ether extract of the saponified neutral lipids. The major steroid (98.5%) was identified as cholesterol with the aid of thin-layer and gas chromatography and mass spectrometry.

The polar lipids were fractionated further by TLC employing two dimensionally development. Figures 1A and 1C compare the total polar lipids in 10 and 26 day old cultures. Considerable degradation of the polar lipids, particularly the phospholipids, had occurred by 26 days. However, several lipids do appear more concentrated in the older mycelia; this probably is due to the resistance of these lipids to catabolism. Figure 1B shows a ^{32}P-labeled extract of *E. coronata* grown for 10 days and is similar to the chromatogram shown in 1A. Figure 1D shows an α-naphthol sprayed chromatogram of the conidial polar lipids. Figure 2 shows the result of four separate sprays (phospray, ninhydrin, 20% sulfuric acid char, and α-naphthol) on thin-layer chromatograms of the polar lipids of 14 day old cultures. A number of polar lipids were observed with a sulfuric acid char but did not give any positive

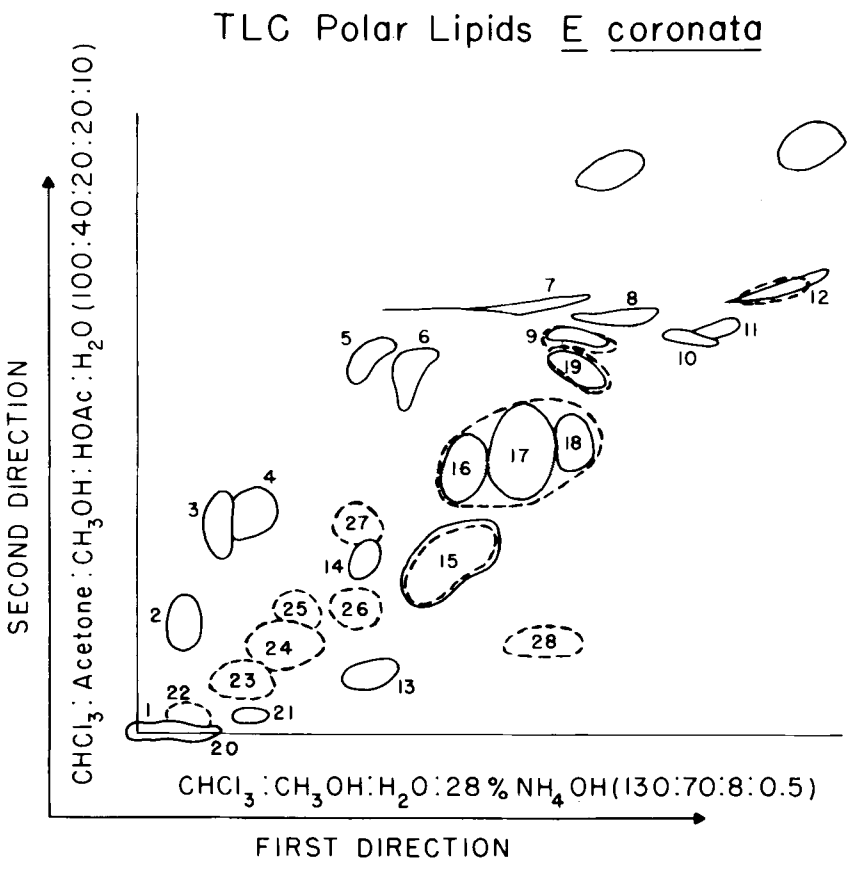

FIG. 3. A composit drawing of all 28 components observed in the polar lipid separations of *Entomophthora coronata*.

functional group spray. Figure 3 shows a composit drawing of all the 28 components observed in the polar lipid separations. A number of the spots observed in the 14 day old cultures are missing in the 26 day old cultures and vice versa.

The phospholipids were identified through cochromatography with standards, by use of selective sprays and by paper chromatography of the individual deacylated ^{32}P-lipids eluted from the thin-layer plates (9). Thus, spots 2, 3, 5, 6, and 9 correspond to lysophosphatidyl choline, sphingomyeline, phosphatidyl choline, lysophosphatidyl ethanolamine, and phosphatidyl ethanolamine, respectively.

Compounds represented by spots 10 and 11 gave a positive Schiff's and α-naphthol spray suggestive of glycolipids. To examine this possibility further, an aliquot of the polar lipids was separated selectively on a silicic acid column (10). The second acetone fraction contained chiefly the glycolipids which separated into three spots on a one dimensional TLC, and they cochromatographed with standard bovine cerebrosides. This fraction was deacylated, according to the procedure of Vance and Sweeley (12). The resulting methyl ester fatty acids were analyzed by GLC, and these results are presented in Table II. The fatty acid composition of these glycolipids is high in 24:0 and 24:1, typical of cerebrosides. The sphingosine bases and the sugar moiety were analyzed simultaneously as their trimethylsilyl derivatives (12). The results of these analyses are shown in Figure 4. A mannitol standard was added as a reference. Only one sugar was observed, galactose. The chromatograms revealed a mixture of nitrogen bases, and their identity will await further investigations.

Table III summarizes the spray results with the 28 designated regions of the chromatogram and their probable identification. Twenty-one compounds still remain to be identified. Spots 13, 15, 20, and 21 gave a positive α-naphthol and Schifff's spray and suggest glycolipids. Several spots also showing intense α-naphthol and Schiff's spray reactions are found in the conidial lipids and also may represent glyco-

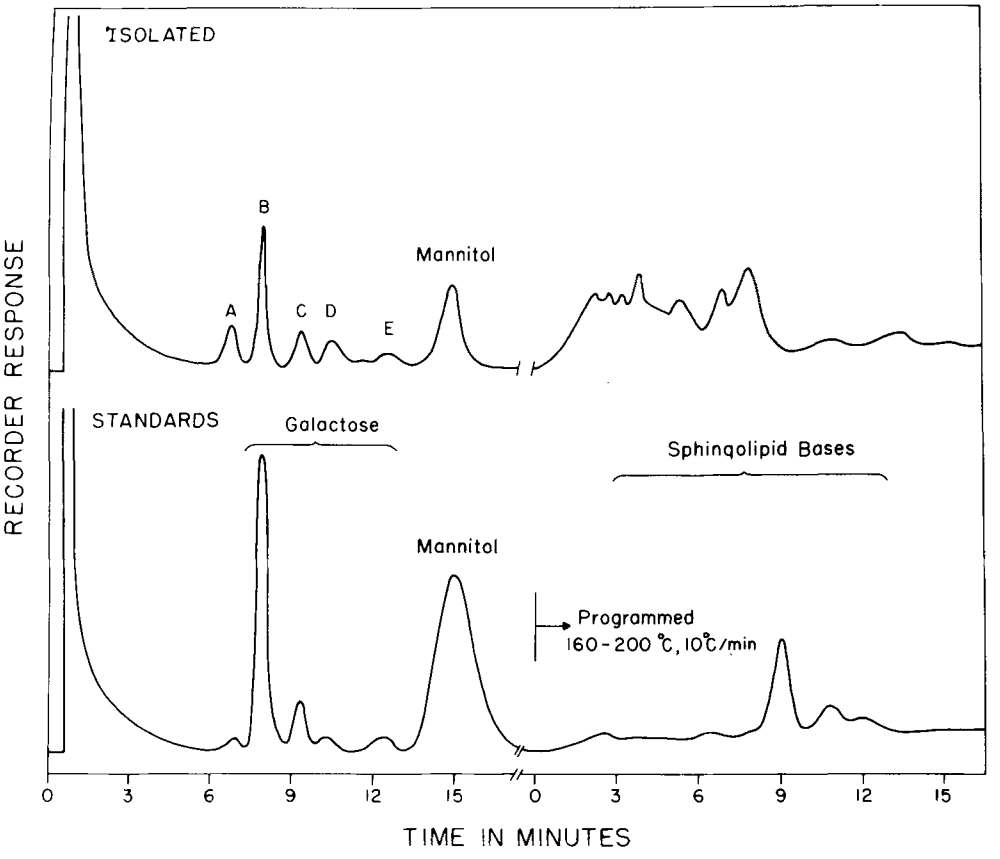

FIG. 4. Gas chromatography of the trimethylsilyl derivatives of the sphingosine bases and the sugar moiety derived from the isolated cerebrosides from *Entomophthora coronata* (spots 10 and 11 of Fig. 3).

lipids. However, the fatty acid profile of the polar lipids of the conidia does not suggest the presence of appreciable glycolipids, since 24:1 is only found in 0.7%.

A number of components give an intense sulfuric acid char but do not give any other positive spray test. Many components were observed with ninhydrin spray; however, most of these spots are not observed with sulfuric acid charring, implying that their concentration is very low. The relative concentration of the cerebrosides increased with age of the culture.

Sphingomyelin, cerebrosides, and cholesterol are lipids typically found in animal tissue and their discovery in significnat quantity in *E. coronata* raises some basic questions. Are the presence of these more animal-like lipids indicative of this organism's ability to parasitize animals? Could animal fungal pathogens have evolved polar lipids typical of animal tissue? Lastly, could the composition of the polar lipids be used to indicate potential animal pathogenicity? This investigation represents, to our knowledge, the only animal fungal pathogen whose lipid composition has been examined in detail, and additional studies on other animal fungal pathogens are needed before any generalizations can be made.

Since Weiss and Stiller recently have reported sphingolipids in mushrooms, in *Phycomycetes blakesleeanus* and in *Fusarium lini* (17,18) perhaps sphingolipids are more widely distributed in fungi than previously realized. In the examined cerebrosides of *E. coronata*, only galactose was identified; and a complex mixture of long chain bases was observed. In comparison, the cerebrosides of *P. blakesleenus* and *F. lini* contained glucose (galactose also in one lipid), hydroxy fatty acids, and a complex mixture of long chain bases.

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REFERENCES

1. Tyrrell, D., Can. J. Microbiol. 13:755 (1967).

2. Tyrrell, D., Botanical Rev. 35:305 (1969).
3. Mumma, R.O., and T.E. Bruszewski, Lipids 5:915 (1970).
4. Bras, G., C.C. Gordon, C.W. Emmons, K.M. Prendegast and M. Sugar, Amer. J. Trop. Med. Hyg. 14:141 (1965).
5. Mumma, R.O., and T.E. Bruszewski, Paper presented in part at the AOCS Meeting, Atlantic City, October 1971.
6. Freeman, C.P., and D. West, J. Lipid Res. 7:324 (1966).
7. Parsons, J.G., and S. Patton, Ibid. 8:696 (1967).
8. Dawson, R.M.C., Biochem. J. 75:45 (1960).
9. Mauro, B., and A.A. Benson, J. Biol. Chem. 234:254 (1959).
10. Rouser, G., G. Kritchevsky, and A. Yamamoto, in "Lipid Chromatographic Analysis," Edited by G.V. Marinetti, Marcel Dekker, New York, N.Y., 1967, pp. 116-120.
11. Siakotos, A.M., and G. Rouser, JAOCS 42:913 (1965).
12. Vance, D.E., and C.C. Sweeley, J. Lipid Res. 8:621 (1967).
13. Folch, J., M. Lees, and G.H. Sloane-Stanley, J. Biol. Chem. 226:497 (1957).
14. Vaskovsky, V.E., and E.Y. Kostetsky, J. Lipid Res. 9:396 (1968).
15. Krebs, K.G., D. Heusser and H. Wimmer. in "Thin-layer Chromatography," Edited by E. Sthal, Academic Press, New York, N.Y., 1969, pp. 485-502.
16. Shaw, N., Biochim. Biophys. Acta 164:435 (1968).
17. Weiss, B., and R.L. Stiller, Biochem. 11:4552 (1972).
18. Weiss, B., and R.L. Stiller, Lipids 8:25 (1973).

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Metabolism of 1,2-(1-^{14}C) Dipalmitoyl Phosphatidylcholine in the Developing Brain[1]

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ABSTRACT

Thirteen-day-old rats were divided into two groups; one group received 1,2-(1-^{14}C) dipalmitoyl phosphatidylcholine and the other 1-^{14}C palmitic acid in the form of an intraperitoneal injection. One half of the total number of rats in each group was sacrificed 24 hr after injection, and the other half was allowed to survive for 17 days after the injection. Radioactivity incorporated into brain and liver total lipids and into individiual polar lipid components of the brain was determined at both intervals. Phosphatidylcholine was isolated and partially deacylated with phospholipase A_2 from *Crotalus Admanteus* venom. The ratio of radioactivity FA 2/FA 1 (fatty acid attached to 2 and 1 carbon of the glycerol moiety) 24 hr after the injection was 8.3, when the tracer was radioactive phosphatidylcholine, compared to only 0.7 when radioactive palmitate was injected. From this different labeling ratio and different pattern of labeling the polar lipid components, it was concluded that the radioactive phosphatidylcholine was not deacylated completely before being taken up directly into the brain. Possibilities are discussed to show that the observed radioactive ratio could result from direct uptake of intact phosphatidylcholine, with little or no restriction from the blood brain barrier system, followed by partial degradation by phospholipase A_1 in the brain itself.

INTRODUCTION

During the past few years, work from this laboratory has shown conclusively that long chain fatty acids (FA), when introduced into the circulating blood, were taken up directly into the brain (1). The much discussed blood-brain barrier system seemed to pose no restriction to this direct uptake. It is possible that, in accordance with Davson's ideas (2), the fatty acids are simply representative of lipid-soluble compounds, which are not restricted by the blood-brain barrier system. On the other hand, many polar compounds with greater water solubility penetrate the system with difficulty (3). The phospholipids, though still lipid-soluble, are considerably more polar than the fatty acids, so the question of restricted penetration of the blood-brain-barrier system could not be answered on the basis of polarity or solubility alone. We, therefore, sought to answer this question using phosphatidylcholine, a compound of greater mol wt and higher polarity than a fatty acid. Hoelzl and Franck (4), who sought information on whether lecithin could penetrate the blood-brain-barrier system, concluded that at least the glyceryl phosphorylcholine backbone penetrated the system. They also found that fatty acids from position 2 were lost somewhere in the process. Illingworth and Portman (5) concluded that plasma lysophosphatidylcholine serves as an important precursor of brain lecithin.

The present study was undertaken to distinguish between two possibilities: first, a direct uptake of intact phosphatidylcholine and, second, a partial or complete degradation of the administered phosphatidylcholine, followed by uptake of components and resynthesis in the brain itself.

MATERIALS AND METHODS

Tracer: 1,2-(1-^{14}C) Dipalmitoyl phosphatidylcholine was purchased from DHOM Products (Hollywood, Calif.); purity was checked by thin layer chromatography (TLC); equal distribution of radioactivity in the 1 and 2 positions of phosphatidylcholine, as well as localization of all radioactivity in the carboxyl carbon of palmitic acid, was ascertained by the methods outlined below.

Animals: Twelve male and female Wistar 13-day old rats weighing ca. 18 g were given intraperitoneal injections of 4 μc 1,2-(1-^{14}C) dipalmitoyl phosphatidylcholine. Six rats were killed 24 hr after the dose, and the other six were allowed to survive for 17 days (average body wt 130 g) before sacrifice. All animals had free access to food (Purina Rat Chow) and water.

Extraction and isolation of lipids: Brain and

[1]Presented in part at the AOCS Meeting, New Orleans, April 1973.

TABLE I

Incorporation of Radioactivity into Brain and Liver Lipids of Rats after Intraperitoneal Injection of 1-^{14}C Dipalmitoyl Lecithin

Tissue	Brain		Liver	
Age of rats (days)	13	30	13	30
Interval between dose and sacrifice	24 hr	17 days	24 hr	17 days
Specific radioactivity (cpm/mg) total lipids	420	125	2,835	18
Radioactivity of total lipids cpm/g wet wt	20,795	11,187	180,774	763
Per cent incorporation $\frac{\text{cpm/g tissue}}{\text{dose/g body wt}} \times 100$	4.2	2.78	36.8	0.16

liver tissues were excised from decapitated animals, washed several times with distilled water, blotted to remove excess water, and placed in tared vials containing chloroform: methanol (2:1 v/v). Lipids were extracted from pooled tissue using the method of Folch, et al., (6). Total lipids were fractionated according to the method described by Rouser, et al., (7). The pure phosphatidylcholine component was subjected to hydrolysis by using Crotalus Admanteus venom (Sigma, St. Louis, Mo.), as described by Van Golde and Van Deenen (8). The resulting lysophosphatidylcholine and fatty acids were separated on a 5 x 20 cm Silica Gel G TLC plate using chloroform: methanol: formic acid: water (70:28:7.5:2.5 v/v) as solvent (9). Immediate radioscanning was performed using a Packard Radiochromatogram Scanner Model 7200. The areas under the recorder peaks were estimated by triangulation. The TLC plate then was exposed briefly to iodine vapor, spots visualized, and areas scraped directly into scintillation vials containing 10 ml Aquasol (New England Nuclear, Boston, Mass.) containing 2 ml water (10). The contents were mixed thoroughly on a Vortex shaker, and radioactivity was determined in a Scintillation Counter (11).

Phosphatidylcholine isolated from the brain and liver total lipids also was hydrolyzed at 37 C using mild alkaline conditions (12). The total fatty acids were methylated with diazomethane. Separation of fatty acid methyl esters was accomplished first by SiO_2:$AgNO_3$ column chromatography and then by preparative gas liquid chromatography (GLC), and the decarboxylation of the resulting pure palmitic and stearic acids was carried out, as described previously (11).

A separate parallel experiment using 1-^{14}C palmitic acid as tracer was performed under identical experimental procedures.

RESULTS

The results of incorporating radioactivity from phosphatidylcholine into the brain and liver lipids of rats sacrificed 24 hr and 17 days after the tracer injection are shown in Table I. The specific radioactivity of brain total lipids 24 hr after injection was lower than that of liver lipids; but, at the end of 17 days, the liver lipids had lost most of the radioactivity, whereas brain lipids retained over 50% original radioactivity.

Brain phosphatidylcholine was degraded partially using venom to obtain the ratio of radioactivity associated with fatty acids at positions 2 and 1 (FA 2/FA 1). The ratio was very high (8.3) 24 hr after injection, indicating relatively greater deacylation of the fatty acids in position 1. At the end of 17 days, the ratio decreased to 2.3 (Table II).

Following injection of 1-^{14}C palmitate, on the other hand, the ratio of radioactivity in FA 2/FA 1 was less than one at both time intervals. This indicates that the radioactive phosphatidylcholine was not deacylated completely before passage into the brain, followed by reacylation in situ.

Phosphatidylcholine also was isolated from the liver total lipids and the ratio FA 2/FA 1 determined (Table II). Irrespective of the two different tracer compounds, the ratio of radioactivities of FA 2/FA 1 was much lower than one. This suggests that the tracer phosphatidylcholine was handled differently by the liver and the brain.

When the total fatty acids were isolated from brain and liver phosphatidylcholine, it was found that, at both time intervals, palmitate was the most radioactive component. Seventeen days after the tracer, radioactivity of stearate increased to a certain extent but mono-, as well as polyunsaturated, fatty acids still had low

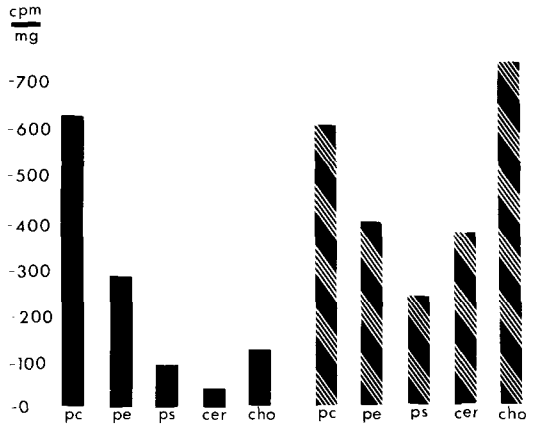

FIG. 1. Specific radioactivity (cpm/mg) of brain polar lipids isolated from 13-day-old rats, 24 hr after tracer. Solid black bars represent value of rats given radioactive phosphatidylcholine. Hatched bars represent values from animals given radioactive palmitic acid. PC = phosphatidylcholine, PE = phosphatidylethanolamine, PS = phosphatidylserine, CER = cerebrosides, and CHO = cholesterol.

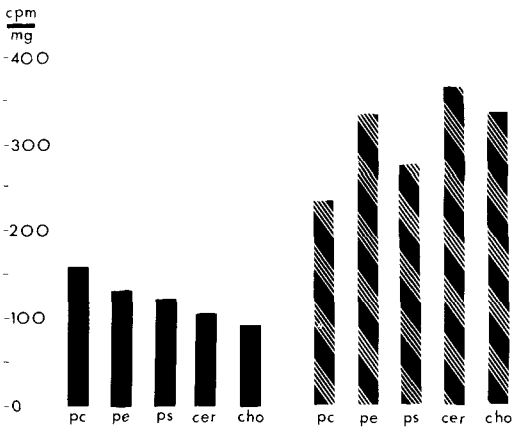

FIG. 2. Specific radioactivity (cpm/mg) of brain polar lipids isolated from rats 17 days after administration of radioactive phosphatidylcholine (solid black bars) and radioactive palmitic acid (hatched bars). Abbreviations same as in Figure 1.

specific activites. To ascertain whether palmitate had undergone any modification, the phosphatidylcholine isolated from both the liver and brain total lipids was hydrolyzed and the purified palmitate decarboxylated (Table III) to determine label distribution. The palmitate both from liver and brain total lipids still retained most of the radioactivity in the carboxyl carbon corroborating our earlier observations (13).

When the specific activities of various polar lipids isolated from pooled brain tissue of rats given radioactive phosphatidylcholine are compared with those of polar lipids obtained from animals given 1-^{14}C palmitate (Fig. 1), differences clearly were observed in the relative patterns of labeling. For example, specific activities of cerebroside and cholesterol components were low when 1-^{14}C dipalmitoyl lecithin was administered, as compared to the high specific activities of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), whereas, if the tracer was 1-^{14}C palmitic acid, it was the cholesterol fraction that had maximum specific radioactivity (higher than PC). At the end of the 17-day period, similar differences were observed (Fig. 2); in addition, phosphatidylcholine isolated from brains of rats given radioactive lecithin had the highest specific activity, unlike the rats given 1-^{14}C palmitic acid. This indicated again that the administered labeled phosphatidylcholine was not deacylated completely before entering the brain.

DISCUSSION

There are several possible transformations that can occur in a heterogenious molecule,

TABLE II

Positional Distribution of Radioactivity in Choline Phosphoglycerides (PC) Isolated from Brain and Liver Tissue

	24 hr after dose			17 days after dose		
	Per cent total radioactivity			Per cent total radioactivity		
Dose administered	Fatty acid 2 position	Lyso PC (FA 1)	Ratio $\frac{\text{FA 2}}{\text{FA 1}}$	Fatty acid 2 position	Lyso PC (FA 1)	Ratio $\frac{\text{FA 2}}{\text{FA 1}}$
Brain						
1-^{14}C dipalmitoyl lecithin	89.3	10.7	8.3	69.6	30.4	2.3
1-^{14}C palmitic acid	41.1	58.9	0.7	45.5	54.5	0.8
Liver						
1-^{14}C dipalmitoyl lecithin	33.7	66.3	0.5	27.2	72.7	0.4
1-^{14}C palmitic acid	18.9	81.1	0.2	13.4	86.6	0.16

TABLE III

Distribution of Radioactivity in Palmitic and Stearic Acids Isolated from Brain and Liver Choline Phosphoglycerides

Age of animals	13 Days				30 Days			
Interval between Dose and sacrifice	24 Hr				17 Days			
Fatty acid	16:0		18:0		16:0		18:0	
	Specific activity cpm/mg	% RCA[a]	Specific activity cpm/mg	% RCA	Specific activity cpm/mg	% RCA	Specific activity cpm/mg	% RCA
Brain	---	93.3	---	23.4	317	81.6	287	12.2
Liver	---	99.9	---	40.4	45	76.4	40	9.1

[a]RCA = relative carboxyl activity $= \frac{\text{radioactivity in COOH} \times 100}{\text{radioactivity in total fatty acid}}$.

such as the 1,2-(1-^{14}C) dipalmitoyl phosphatidylcholine that was used as a tracer. Consider, for example, that it was deacylated completely by the combined action of various phospholipases. The fatty acyl components released then could be taken up directly into the brain, as shown earlier (1), and reacylated. This possibility could be simulated by injecting 1-^{14}C palmitate as tracer. However, the experimental values show that the ratio FA 2/FA 1 in terms of radioactivity is quite different when the two tracers were used. Under identical experimental conditions, the two tracers resulted in a different pattern of relative radioactive specific activites (cpm/mg) of brain polar lipids (Fig. 1). It is assumed that there is a common pool of palmitate both arising from deacylation of lecithin and entering as free palmitate. Although it is not known that this is the case, definite experimental evidence showing different pools of any one particular fatty acid has not, to our knowledge, been documented. In our earlier work (13), it was found that acetate, derived from breakdown of palmitate, was utilized for chain elongation and cholesterol synthesis but not for resynthesis of palmitate, although exogenously administered acetate was utilized equally well for all reactions (11). Thus it seems clear that the tracer radioactive phosphatidylcholine was not completely deacylated before entering the brain.

Another possibility is that the radioactive phosphatidylcholine may be partially degraded. For example, the plasma lecithin: cholesterol: acyltransferase (14) could form 1-acyl lysophosphatidylcholine. This compound could enter the brain easily, as shown by Illingworth and Portman (5), and be reacylated. The reacylation of the lyso compound could occur with a variety of fatty acyl moieties. According to Baker and Thompson (15), palmitate and stearate are incorporated preferentially into position 1 and thus are excluded from position 2. However, Montfoort et al., (16) analyzed the phosphatidylcholine species from many different organs and reported that the predominant species of rat brain phosphatidylcholine are 1-palmitoyl-2-oleoyl (ca. 30%) and dipalmitoyl lecithin (ca. 15%). If we assume that the partially deacylated tracer was reacylated with the oleoyl moiety, then the ratio of radioactivity FA 2/FA 1 would be less than one. If the reacylation reaction used the palmitoyl group, the ratio would be one or lower, if we assume a decrease in radioactivity of palmitate from dilution by the brain palmitate pool. Arachidonate has been shown to be incorporated into position 2 (5,15), but in the present study 20:4 had negligible radioactivity and so the FA 2/FA

1 ratioactivity ratio would be very low. Since the experimental value of 8.3 exceeds any of the values in these considerations, partial degradation to form 1-acyl lysophosphatidylcholine may be ruled out.

Using the same argument as the above, one can rule out the possiblility of partial degradation of the tracer by phospholipase A_2 from either brain or other tissues because the A_2 action will again result in formation of 1-acyl lysolecithin.

One can consider yet another possibility of partial degradation of the tracer by phospholipase A_1 leading to the formation of 2-palmitoyl lysolecithin in the liver followed by uptake into the brain. The reacylation of position 1 in the liver lecithin species would be largely with saturated acids (16), such as palmitate or stearate. The palmitate, now diluted by the endogenous pool, would give a species in which the radioactivity ratio FA 2/FA 1 would be higher than one. (The same would be true if stearate were used in reacylation). Since the observed FA 2/FA 1 ratio in the liver lecithin was less than one, this possibility also may be ruled out. Phospholipase A_1, as well as A_2, activity in extrahepatic tissue, such as adrenal medula (17), spleen (18), red blood cells (19), and even plasma (20), has been reported. Such activity (18-20) was determined using phosphatidylglycerol and not phosphatidylcholine as substrate. The magnitude and physiological impact of such enzyme activity from these extrahepatic tissue is not yet established. However, one has to consider the possiblity of such extrahepatic tissue phospholipase A_1 influencing the nature of the radioactive compound entering the brain. Lysolecithinase (lysolecithin acyl hydrolase E·C·3·1·1·5) obtained from rat brain microsomal fraction and purified 20 times has been characterized by Leibovitz and Gatt (21). This enzyme hydrolyzes 1-acyl-sn-glycero-3-phosphoryl-choline (product of phospholipase A_2) and as such could influence the distribution of radioactivity in the brain lecithin.

The last possiblility to consider is that radioactive phosphatidylcholine was taken up directly into the brain followed by action of phospholipase A_1 in the brain itself. This would give a 2-acyl lysophosphatidylcholine which would be reacylated with palmitate (with reduced radioactivity from dilution) or with stearate (15,16). In all cases, the ratio of radioactivity of FA 2/FA 1 would be much greater than one. The experimental value was indeed much higher than one, indicating direct uptake of the tracer phosphatidylcholine, specifically 1,2, dipalmitoyl lecithin in our experiments, followed by phospholipase A_1 action in the brain itself. The occurrence of phospholipase A_1 in the rat brain has been demonstrated by Gatt (22).

Even at the end of the 17-day period, the radioactivity ratio FA 2/FA 1 in animals given radioactive phosphatidylcholine was much higher than in animals given radioactive palmitate. Therefore during this period, complete deacylation-reacylation does not seem to have taken place. It is possible to speculate that developing brain may change its phospholipase enzyme specificity from A_1 to A_2, in which case the radioactivity ratio FA 2/FA 1 will remain higher than one but much lower than that obtained at the end of 24 hr. However, no direct evidence for this was obtained in the present study.

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REFERENCES

1. Dhopeshwarkar, G.A., C. Subramanian, D.H. McConnel and J.F. Mead, Biochim. Biophys. Acta. 255:572 (1972).
2. Davson, H., Advan. Exp. Med. Biol. 13:381 (1971).
3. Oldendorf, W.H., Amer. J. Physiol. 221:1629 (1971).
4. Hoelzl, J., and H.P. Franck, Second International Meeting, International Society Neurochemistry, September 2, 1969, Milan, p. 219.
5. Illingworth, D.R., and O.W. Portman, Biochem. J. 130:557 (1972).
6. Folch, J., M. Lees and G.H. Sloane-Stanley, J. Biol. Chem. 226:497 (1957).
7. Rouser,, G., G. Kritchevsky, D. Heller and E. Lieber, JAOCS 40:425 (1963).
8. Van Golde, L.M.G., and L.L.M. Van Deenen, Biochim. Biophys. Acta. 125:496 (1966).
9. Wells, M.A., and D.J. Hanahan in "Methods of Enzymology," Vol. 14, Edited by J.M. Lowenstein, Academic Press, New York, N.Y., 1969, p. 178.
10. Webb, R.A., and D.F. Mettrick, J. Chromatog. 67:75 (1972).
11. Dhopeshwarkar, G.A., R. Maier and J.F. Mead, Biochim. Biophys. Acta. 187:6 (1969).
12. Dawson, R.M.C., Ibid. 14:374 (1954).
13. Dhopeshwarkar, G.A., C. Subramanian and J.F. Mead, Ibid. 296:57 (1972).
14. Glomset, J.A., J. Lipid Res. 9:155 (1968).
15. Baker, R.R., and W. Thompson, Biochim. Biophys. Acta. 270:489 (1972).
16. Montfoort, A., L.M.G. Van Golde and L.L.M. Van Deenen, Ibid. 231:335 (1971).
17. Smith, A.D., and H. Winkler. Biochem. J. 108:867 (1968).
18. Paysant, M., R. Wald and J. Polonovsky, Bull.

Soc. Chim. Biol. 50:1445 (1968).
19. Paysant, M., D. Delbauffe, R. Wald and J. Polonovsky, Ibid. 49:169 (1967).
20. Paysant, M., and J. Polonovsky, C.R. Acad. Sci. (Paris) 263:1419 (1966).
21. Leibovitz, Z., and S. Gatt, Biochim. Biophys. Acta. 164:439 (1968).
22. Gatt, S., Ibid. 159:304 (1968).

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Phospholipids of the Pulmonate Land Snail *Cepaea nemoralis* (L.)

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ABSTRACT

The phospholipids of the snail *Cepaea nemoralis*, comprising the major lipid fraction (65%) in this terrestrial pulmonate, were investigated by thin-layer and column chromatography. Detailed gas chromatographic analyses of liberated fatty acid fractions and amino acid analyses of the water soluble moieties of isolated phospholipid classes were carried out. Phosphatidyl choline (47%) and phosphatidyl ethanolamine (21%) were found to be the predominant phospholipid classes, while phosphatidyl serine (8%), phosphatidyl inositol (6%), diphosphatidyl glycerol (3%), ceramide aminoethylphosphonate (7%), lysophosphatidyl choline (1%), and phosphatidic acid (1%) were present in lesser amounts. In the phosphatidyl ethanolamine and phosphatidyl serine fractions, minor quantities of plasmalogen analogues were detected. Fatty acid profiles of the various phospholipid classes appeared to be strikingly diverse, e.g. a characteristic component, such as linoleic (18:2ω6) acid, ranging from 3-54%. In vivo radioisotope studies using 1-^{14}C-acetate demonstrated the high biosynthetic rate of all phospholipid classes and their respective fatty acid fractions. Results are discussed in relation to data on the phospholipids from other invertebrate species.

INTRODUCTION

Molluscan phospholipids have been studied rather intensively with respect to some interesting compounds which are relatively abundant, particularly in molluscs.

Thus, the phosphonolipid ceramide aminoethylphosphonate (CAEP), originally discovered by Rouser, et al., (1) in the sea anemone *Anthopleura elegantissima*, appears to be distributed widely within the phylum Mollusca (2-10), while more recently other sphingophosphonolipids have been characterized in molluscs too (11-13). Moreover, molluscs are very rich in ether-containing phospholipids, both plasmalogens and α-glyceryl ethers (14-17); and two species of slug have since been used in studies on ether-lipid metabolism (17-20).

From our investigations into the lipid composition and metabolism of the land snail *Cepaea nemoralis* (21-27), it became evident that, in this species, phospholipids constituted the major lipid fraction (ca. 65% total lipids), which actively was synthesized from several radiolabeled precursors. Therefore, in this report we present: (a) the qualitative and quantitative phospholipid composition of *C. nemoralis*, (b) the detailed fatty acid composition of seven phospholipid classes, and (c) data on the in vivo biosynthesis of the phospholipids from 1-^{14}C-acetate.

MATERIALS AND METHODS

Experimental animals of the species *C. nemoralis* (L.) (Gastropoda:Pulmonata) were caught in June 1970 in the vicinity of Utrecht. Snails (350) were each injected with 1 μl aqueous solution of sodium-1-^{14}C-acetate (New England Nuclear, Dreieichenhain, W. Germany, spec. act. 2 mCi/mmol). Incubation was terminated after 24 hr by deep-freezing (21).

Isolation of Phospholipids

Upon removal of the shells, the animals (584 g) were homogenized in a Sorvall Omnimixer with chloroform-methanol, thereafter total lipids were extracted, according to van der Horst, et al., (28) and purified by Sephadex G-25 column chromatography (29).

Since ca. 60% total fatty acids of *C. nemoralis* is polyunsaturated (21,25) and consequently extremely sensitive to oxidation during lipid fractionation, an evaluation of some current phospholipid isolation procedures was essential to obtain a reliable composition of the total phospholipid fatty acids, which was to be used as a control for the fatty acid compositions of the different phospholipid classes.

Therefore, total phospholipids were isolated by: (a) repeated precipitation with acetone-$MgCl_2$ at -28 C (30); (b) column chromatography on silicic acid (Mallinckrodt, 100 mesh) mixed with Hyflo Super-Cel (Johns Manville, New York, N.Y.) (31); and (c) column chromatography on diethylaminoethyl (DEAE) cellulose (32). To prevent lipid oxidation in all procedures, an atmosphere of nitrogen was provided where possible. Freshly redistilled reagent grade organic solvents containing 10 ppm BHT

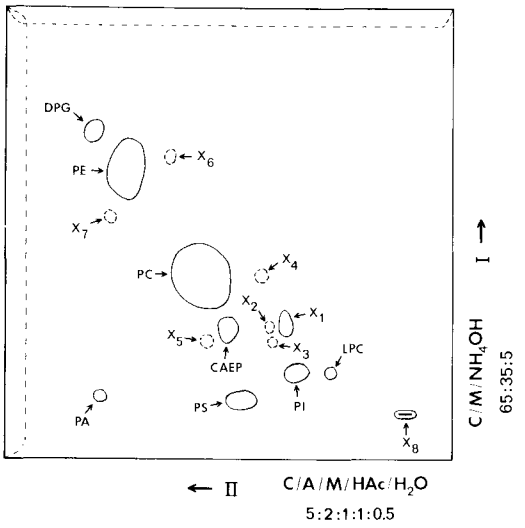

FIG. 1. Two-dimensional thin-layer chromatogram of phospholipids of *Cepaea nemoralis*. The sample was applied in the lower right corner (X_8). In the first dimension chloroform/methanol/28% aqueous ammonia 65:35:5 was used, followed by development in the second dimension with chloroform/acetone/methanol/acetic acid/water 5:2:1:1:0.5. Abbreviations: PE = phosphatidyl ethanolamine, PC = phosphatidyl choline, PS = phosphatidyl serine, PI = phosphatidyl inositol, PA = phosphatidic acid, CAEP = ceramide aminoethylphosphonate, DPG = diphosphatidyl glycerol (cardiolipin), and LPC = lysophosphatidyl choline. Minor unidentified phospholipids are designated X_1-X_8.

(butylated hydroxy toluene = 2,6-di-*tert*-butyl *p*-cresol) were used. Lipid samples were stored in acetone containing 100 ppm BHT in an atmosphere of nitrogen at -28 C in the dark.

Qualitative Analysis of Phospholipids

By two-dimensional thin-layer chromatography (TLC), according to the procedure of Rouser, et al., (7,33), phospholipid classes were separated and identified based upon their characteristic chromatographic properties and reactivity to spray reagents, such as ninhydrin (amino-lipids), Dragendorff's reagent (choline), molybdenum blue (phospholipids) (34), ammoniacal silver nitrate (inositol), Schiff's reagent (aldehyde groups), hydroxylamine-ferric chloride (esterified fatty acids), and Rhodamine 6G (nonspecific), in comparison with pure reference substances commercially available for most phospholipid classes.

Determination of Plasmalogens

The two-dimensional TLC procedure of Rouser, et al., (7,33) also was applied for the determination of plasmalogens. After developing a chromatogram in the first dimension, the right-hand lipid tract was sprayed with a solution of 5 mM mercuric chloride in 0.1 M acetic acid (35), while the remainder of the plate was screened. After drying by evacuation, the $HgCl_2$-treated lipids were developed in the second dimension and visualized by the detection reagents, already mentioned. Additionally, chromatograms were sprayed with a colorless mercury-sensitive reagent (36) to detect the β-chloromercuri-aldehydes derived from plasmalogens by the treatment with $HgCl_2$ (37).

Quantitative Phosphorus Determination

The phosphorus content of spots isolated after two-dimensional TLC was measured at 830 nm with a Zeiss PMQ II spectrophotometer, according to Böttcher, et al. (38).

Preparative Isolation of Phospholipid Classes

Samples of the total phospholipids were fractionated by column chromatography on silicic acid-Hyflo Super-Cel (31). The individual phospholipid classes were purified further by preparative one-dimensional TLC on plates coated with silicagel H (Merck) in 0.1 mM sodium carbonate (thickness of adsorbent 0.5 mm), employing chloroform/methanol/28% aqueous ammonia/water 67:33:3:2 or 70:30:3:2 as the solvent system. Purities of all isolated phospholipid classes were checked by two-dimensional TLC.

Isolation and Analysis of Phospholipid Fatty Acids

Samples of total phospholipids and isolated phospholipid classes were saponified in methanolic KOH (21), except for CAEP, which was acid hydrolyzed (39). Fatty acids were isolated, methylated with diazomethane, and purified fatty acid methylesters were separated into saturated and unsaturated fractions, as described elsewhere in more detail (21,40). Analytical gas liquid chromatography (GLC) of fatty acid methylester samples was performed on two different stationary phases (40,41).

Determination of Phospholipid Amino Acids

Water soluble components from hydrolyzed total phospholipid samples and isolated phosphatidyl serine fractions were assayed for amino acids with a Biocal BC-200 automatic amino acid analyzer using a Bio-Rad Aminex A-6 cation exchanger and 0.3 N lithium citrate buffers.

Liquid Scintillation Spectrometry

Radioactivities of all lipid samples were measured in toluene-Omnifluor (New England Nuclear), using a Packard Model 2420 spec-

TABLE I

Phospholipid Composition of *Cepaea nemoralis*

Phospholipid class	% total phosphorus[a,b]
Phosphatidyl choline	47.0 ± 0.1
Phosphatidyl ethanolamine	20.7 ± 0.1
Phosphatidyl serine	8.1 ± 0.2
Phosphatidyl inositol	5.6 ± 0.1
Diphosphatidyl glycerol	3.2 ± 0.2
Ceramide aminoethylphosphonate	6.8 ± 0.3
Lysophosphatidyl choline	1.1 ± 0.3
Phosphatidic acid	0.6 ± 0.1
X_1	5.6 ± 0.3
X_8	1.2 ± 0.3

[a]Mean values of three trials ± standard deviation.

[b]Phosphorus content of X_2-X_7 was under detection limit.

trometer. Direct radioassay of the amino acids eluted from the amino acid analyzer was performed with a Packard Model 2002 instrument equipped with an anthracene flow cell.

RESULTS

Phospholipid Isolation and Distribution

Lipid extraction yielded 7.92 g purified total lipids (1.36% the fresh wt) with a specific radioactivity of 2729 dpm/mg. Data from the three phospholipid isolation procedures showed that phospholipid content of total lipids was in the range of 63.2-64.6% by wt, the specific radioactivity being 3279-3328 dpm/mg. Phosphorus content of total phospholipids amounted to 3.8%. Purities of phospholipids and neutral lipids (including free fatty acids) were checked by analytical TLC. Only in the acetone-precipitated phospholipids, a trace of sterol was present, which was removed easily by silicic acid column chromatography using chloroform as the eluent. All isolated neutral lipid fractions were free of phosphorus-containing compounds. A typical TLC separation of phospholipid classes is depicted in Figure 1, while the quantitative phospholipid distribution determined by phosphorus analysis of the spots shown in Figure 1 is presented in Table I.

The phospholipids of the snail are composed of a large number of classes. Serine appeared to be the only amino acid present in the phospholipids and was associated with phosphatidyl serine. Minor proportions (ca. 10-15%) of phosphatidyl ethanolamine and phosphatidyl serine were identified as plasmalogens, since, after spraying with mercuric chloride, lysophosphatidyl ethanolamine and lysophosphatidyl serine were demonstrated. As the mercury-sensitive reagent stained the entire solvent front rather unspecifically, the β-chloromercuri-aldehydes derived from these plasmalogens were less easy detectable. The presence of CAEP also was confirmed by acid hydrolysis of the purified fraction, yielding sphingosine and fatty acid methylesters (39,42).

In determining the fatty acid compositions of isolated total phospholipid fractions, the so far remarkable conformity in the data from the

TABLE II

Proportional Distribution of the Radiolabel from 1-^{14}C-Acetate over the Phospholipid Classes of *Cepaea nemoralis*

Phospholipid class	% total phospholipid radioactivity
Phosphatidyl choline	47.6
Phosphatidyl ethanolamine	19.3
Phosphatidyl serine	7.4
Phosphatidyl inositol	6.5
Diphosphatidyl glycerol	2.0
Ceramide aminoethylphosphonate	6.1
Lysophosphatidyl choline	1.2
Phosphatidic acid	0.7
X_1	2.4
X_8	1.0
Rest (X_2-X_7)	5.8

TABLE III

Specific Radioactivities (in dpm/mg) of the Major Snail Phospholipid Classes and their Fatty Acids

Fraction	Total	Fatty acid methylesters	Saturated fatty acid methylesters	Unsaturated fatty acid methylesters
Total phospholipids	3279	4518	---	---
Phosphatidyl choline	3379	5230	9232	4277
Phosphatidyl ethanolamine	3336	4192	6782	3277
Phosphatidyl serine	3161	6034	---	---
Phosphatidyl inositol	3640	3902	---	---
Diphosphatidyl glycerol	1926	3155	---	---
Ceramide aminoethylphosphonate	3521	—	---	---
Lysophosphatidyl choline	2935	4892	---	---

column chromatographic and precipitation isolation techniques was discontinued. The content of polyunsaturated fatty acids, particularly tetraenoic ones, such as arachidonic (20:4ω6), of the phospholipids isolated by column chromatography was reduced substantially (up to 23%). Therefore, acetone-precipitated total phospholipid fractions were preferred for further investigation.

Distribution of Radioactivity over the Phospholipid Classes

The proportional incorporation of 1-^{14}C-acetate into the different phospholipid classes was determined by radioassaying the scraped spots obtained after two-dimensional TLC of the total phospholipids isolated by acetone precipitation. Mean values from three analyses (corrected for background and counting efficiency) are given in Table II. The toluene-Omnifluor scintillation medium contained 4% aerosil. All phospholipid classes appeared to be radiolabeled (Table II); and, besides, the distribution of carbon-14 over these classes is remarkably identical to that of phosphorus (Table I).

Specific Radioactivity of Phospholipid Classes and their Fatty Acid Fractions

Seven major phospholipid classes were isolated by column chromatography and additional purification by preparative TLC. Specific radioactivities of these classes were determined, as well as of their fatty acid fractions, provided that sufficient material was available (Table III).

Apart from diphosphatidyl glycerol, apparently no marked differences were noticed in the specific radioactivities of the various phospholipid classes (Table III), as already was to be expected on account of the data summarized in Tables I and II. Specific radioactivities of the phospholipid fatty acid fractions, however, show rather divergent values (Table III), which may be attributed to differences in the fatty acid compositions of the phospholipid classes and thus will be discussed in connection with the GLC data. The high specific radioactivity of the fatty acids from phosphatidyl serine compared to the value for total phosphatidyl serine may be explained by the content of normally low-labeled plasmalogens (18,19). Moreover, in the serine moiety, which was radioassayed during amino acid analysis, no carbon-14 incorporation was detected at all. The metabolic predominance of saturated fatty acid biosynthesis over that of unsaturated ones agrees with our earlier studies (24,26,27).

Phospholipid Fatty Acid Compositions

The detailed fatty acid compositions of the seven major phospholipid classes are given in Table IV. For ease of survey, a summary of these compositions is inserted in Table V.

From Tables IV and V it is clear that the fatty acid compositions of the various phospholipid classes are strikingly different, unsaturated fatty acid content ranging from 46% in CAEP up to 83% in diphosphatidyl glycerol. Therefore, it needs no further argumentation that specific radioactivities of the fatty acid fractions of the various phospholipid classes are likewise widely divergent (Table III).

DISCUSSION

In *Cepaea*, the choline and ethanolamine phosphoglycerides appeared to be the main phospholipids, comprising 47% and 21% of the lipid phosphorus, respectively. In addition, phosphatidyl serine (8%), phosphatidyl inositol (6%), diphosphatidyl glycerol (3%), CAEP (7%), lysophosphatidyl choline (1%), and phosphatidic acid (1%) were present, as well as a number of minor unidentified phospholipid components (X_2-X_8 in Figure 1). X_1, being a more prominent component (6%), may be identified tentatively as lysophosphatidyl etha-

TABLE IV

Proportional Fatty Acid Composition (in mol %) of the Major Phospholipids of *Cepaea nemoralis*[a]

Fatty acid	Phospholipid class[b]						
	PC	PE	PS	PI	DPG	LPC	CAEP
<14:0	0.43	0.05	0.23	0.12	0.51	0.45	0.18
14:0	0.58	0.54	---	0.36	1.13	1.09	0.21
14:1	---	0.02	0.07	0.19	---	---	---
14:2	0.08	0.02	---	---	---	---	0.17
br15:0[c]	0.62	0.43	0.08	0.15	0.27	1.04	0.44
15:0	0.76	0.62	0.12	0.23	0.31	0.85	0.28
15:1	0.05	0.20	---	---	0.12	---	0.21
15:2	0.10	0.12	---	0.20	---	---	---
br16:0	0.26	2.42	0.24	0.31	0.28	0.84	0.45
16:0	6.20	3.07	1.76	2.31	2.54	8.15	23.22
16:1	0.20	0.84	0.14	0.22	0.62	0.98	---
16:2	Trace	Trace	---	---	---	---	---
br17:0	1.85	0.47	0.46	1.01	0.82	2.49	1.40
17:0	1.47	1.29	1.35	1.16	0.45	1.76	1.91
17:1	0.20	0.45	0.26	---	0.29	---	---
17:2	Trace	---	---	---	---	---	---
br18:0	1.61	0.86	0.05	0.34	0.15	0.65	0.35
18:0	3.12	11.25	34.94	24.56	3.86	3.97	10.66
18:1	16.60	2.41	3.10	2.95	3.38	10.53	6.84
18:2ω6	13.00	8.68	3.14	5.18	53.95	12.38	3.03
18:3ω3	2.10	1.67	0.52	1.04	11.92	1.11	0.58
br19:0	1.21	2.10	1.77	1.66	0.37	3.24	0.81
19:0	0.09	0.40	1.75	1.30	---	---	---
19:1	0.70	0.50	0.68	0.60	0.43	1.20	0.98
br20:0	0.05	---	0.26	0.46	0.52	0.96	0.93
20:0	0.20	0.79	1.53	1.17	0.64	---	---
20:1	2.51	3.39	5.81	2.83	1.05	2.37	2.75
20:2ω6	15.23	12.92	1.75	5.16	4.52	11.60	1.66
20:3ω6	1.97	2.92	1.33	0.66	0.30	4.22	0.63
20:4ω6	13.70	22.07	29.43	34.09	4.69	9.44	10.71
20:4ω3	---	0.56	---	---	---	---	---
20:5ω3	0.30	1.63	0.96	3.90	---	1.47	2.75
br21:0	0.16	0.22	---	---	---	---	3.04
21:0	0.22	Trace	0.60	0.74	---	---	0.60
21:1	---	---	---	---	0.52	---	---
br22:0	0.22	0.12	0.37	0.99	0.66	2.08	2.00
22:0	Trace	Trace	---	---	1.05	---	---
22:1	1.30	3.95	3.26	0.67	0.62	1.46	1.00
22:3ω6	0.96	2.78	0.66	0.99	---	---	5.88
22:4ω6	6.71	5.76	1.84	1.61	---	6.20	7.61
22:5ω6	1.33	0.41	---	0.71	0.50	---	---
22:5ω3	2.21	1.40	---	---	---	5.66	1.50
br23:0	0.26	---	---	0.24	0.50	0.54	1.48
23:0	Trace	0.53	---	---	---	---	0.10
br24:0	Trace	0.52	0.21	1.01	0.94	1.42	2.98
24:0	Trace	---	0.22	0.61	0.40	0.79	1.52
24:1	---	---	0.46	---	---	---	---
24:2	0.36	0.24	---	---	---	---	---
24:4ω6	0.27	0.73	---	---	0.54	1.06	---
24:5	---	0.83	---	---	---	---	---
br25:0	0.32	---	0.63	---	0.72	---	---
br26:0	0.21	0.37	---	0.30	0.34	---	---
26:0	0.39	---	---	---	---	---	---

[a]Presentation of gas liquid chromatography data to two decimal places is solely to show low proportions of minor components (43). Mean values of three analyses are given.

[b]Abbreviations identified in Figure 1.

[c]Total values for iso-, anteiso-, and internally monomethyl branched fatty acids are given. br = branched (*chain*).

TABLE V

Summary of the Fatty Acid Composition of the Major Phospholipids of *Cepaea nemoralis*[a]

Fatty acids	Phospholipid class[b]						
	PC	PE	PS	PI	DPG	LPC	CAEP
Saturated straight-chain	13.03	17.96	42.04	32.44	10.38	16.61	38.50
Saturated branched-chain	6.77	7.51	4.07	6.47	5.57	13.26	15.50
Monoenoic	21.56	11.76	13.78	7.46	7.03	16.44	11.78
Dienoic	28.77	21.98	4.89	10.54	58.47	23.98	4.86
Trienoic	5.03	7.37	2.51	2.69	12.22	5.33	7.09
Tetraenoic	20.68	29.12	31.27	35.70	5.23	16.64	17.78
Pentaenoic	3.84	4.27	0.96	4.61	0.50	7.13	4.25
Total saturated	19.80	25.47	46.11	38.91	15.95	29.87	54.00
Total unsaturated	79.88	74.55	53.64	61.00	83.45	69.52	45.76

[a]Fatty acids eluted before myristic (14:0) acid are not included. Figures are calculated from Table IV.

[b]Abbreviations identified in Figure 1.

nolamine.

The pattern of phospholipid distribution in this species compares generally to the work from other laboratories on the phospholipids of several molluscs, such as *Lymnaea stagnalis* (8) and other pulmonate snails (15), and even some marine species like abalone (7,44), scallop (7), and whelk (45).

Plasmalogen analogues of diacylphospholipids were detected only in phosphatidyl ethanolamine and phosphatidyl serine. Similar results were obtained for other molluscan species (19,44,46). The content of plasmalogens has been suggested to be subject to seasonal variation (47). The presence of CAEP in *C. nemoralis* and the apparent absence of sphingomyelin may be viewed in the light of a compensatory correlation of their amounts (5,42,44).

However, little is known about the fatty acid composition of phospholipid classes in molluscs. Besides, in the phosphoglycerides saturated ether side chains might be present (14-17), which are found normally in the lipid molecules at positions otherwise occupied by saturated fatty acids, thus influencing the fatty acid compositions. Hence, an interspecies comparison of the complex data obtained for *Cepaea* is hardly realizable.

The snail CAEP fatty acids are characterized by relatively high percentages for palmitic and stearic acid (Table IV), which conforms to CAEP fatty acid compositions from other invertebrates (6,42,44,48).

In diphosphatidyl glycerol the abundancy of C_{18} polyunsaturated fatty acids (66%) is overwhelming. Gray and Macfarlane (49) provided similar results for diphosphatidyl glycerol isolated from ox heart. These specific compositions may have an as yet unproven physiological significance.

In most phosphoglycerides polyunsaturated fatty acids, such as linoleic (18:2ω6), homolinoleic (20:2ω6), and arachidonic (20:4ω6), are important constituents, as was to be expected for the structural lipids of a terrestrial organism (21,24,25). Fatty acid fractions of all phospholipid classes were highly radiolabeled from 1-^{14}C-acetate, indicating a very active biosynthesis. It is noteworthy that this phenomenon is deviating appreciably from the findings of Shieh (50), who supposed a dietary origin of scallop fatty acids on account of the nonlabeling of phospholipid fatty acids after injection of 1-^{14}C-acetate.

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REFERENCES

1. Rouser, G., G. Kritchevsky, D. Heller and E. Lieber, JAOCS 40:425 (1963).
2. Hori, T., O. Itasaka, H. Inoue and K. Yamada, J. Biochem. (Tokyo) 56:477 (1964).
3. Hori, T., O. Itasaka, H. Inoue and M. Akai, Jap. J. Expt. Med. 35:81 (1965).
4. Hori, T., I. Arakawa and M. Sugita, J. Biochem. (Tokyo) 62:67 (1967).
5. Hori, T., O. Itasaka, M. Sugita and I. Arakawa, Mem. Fac. Educ. Shiga Univ. 17:23 (1967).
6. Higashi, S., and T. Hori, Biochim. Biophys. Acta 152:568 (1968).
7. Simon, G., and G. Rouser, Lipids 4:607 (1969).
8. Liang, C-R., and K.P. Strickland, Can. J. Biochem. 47:85 (1969).
9. Liang, C-R., M. Segura and K.P. Strickland, Ibid. 48:580 (1970).
10. Voogt, P.A., in "Chemical Zoology," Vol. 6, Edited by M. Florkin and B.T. Scheer, Academic Press, New York, N.Y., 1972, p. 245.
11. Hori, T., and I, Arakawa, Biochim. Biophys. Acta 176:898 (1969).

12. Hayashi, A., F. Matsuura and T. Matsubara, Ibid. 176:208 (1969).
13. Hayashi, A., and F. Matsuura, Ibid. 248:133 (1971).
14. Karnovsky, M.L., W.S. Rapson and M. Black, J. Soc. Chem. Ind. 65:425 (1946).
15. Thompson Jr., G.A., and D.J. Hanahan, J. Biol. Chem. 238:2628 (1963).
16. Thompson Jr., G.A., and P. Lee, Biochim. Biophys. Acta 98:151 (1965).
17. Thompson Jr., G.A., in "Ether Lipids," Edited by F. Snyder, Academic Press, New York, N.Y., 1972, p. 313.
18. Thompson Jr., G.A., J. Biol. Chem. 240:1912 (1965).
19. Thompson Jr., G.A., Biochemistry 5:1290 (1966).
20. Thompson Jr., G.A., Biochim. Biophys. Acta 152:409 (1968).
21. van der Horst, D.J., Neth. J. Zool. 20:433 (1970).
22. van der Horst, D.J., and R.C.H.M. Oudejans, Comp. Biochem. Physiol. 41B:823 (1972).
23. van der Horst, D.J., and P.A. Voogt, Ibid. 42B:1 (1972).
24. van der Horst, D.J., Ibid. 46B:551 (1973).
25. van der Horst, D.J., and D.I. Zandee, J. Comp. Physiol. 85:317 (1973).
26. van der Horst, D.J., Comp. Biochem. Physiol., In press.
27. Oudejans, R.C.H.M., and D.J. van der Horst, Ibid., In press.
28. van der Horst, D.J., A.H. van Gennip and P.A. Voogt, Lipids 4:300 (1969).
29. Terner, C., E.I. Szabo, and N.L. Smith, J. Chromatog. 47:15 (1970).
30. Lipsky, S.R., A. Haavik, C.L. Hopper and R.W. McDivitt, J. Clin. Invest. 36:233 (1957).
31. Hanahan, D.J., J.C. Dittmer and E. Warashina, J. Biol. Chem. 228:685 (1957).
32. Rouser, G., G. Kritchevsky and A. Yamamoto, in "Lipid Chromatographic Analysis," Vol. 1, Edited by G.V. Marinetti, Marcel Dekker, New York, N.Y., 1967, p. 99.
33. Rouser, G., A.N. Siakotos and S. Fleischer, Lipids 1:85 (1966).
34. Dittmer, J.C., and R.L. Lester, J. Lipid Res. 5:126 (1964).
35. Broekhuyse, R.M., Ph.D. Thesis, University of Nijmegen, Nijmegen, The Netherlands.
36. Owens, K., Biochem. J. 100:354 (1966).
37. Norton, W.T., Nature, London 184:1144 (1959).
38. Böttcher, C.J.F., C.M. van Gent and C. Pries, Anal. Chim. Acta 24:203 (1961).
39. Gaver, R.C., and C.C. Sweeley, JAOCS 42:294 (1965).
40. van der Horst, D.J., R.C.H.M. Oudejans, A.G. Plug and I. van der Sluis, Mar. Biol. 20:291 (1973).
41. Oudejans, R.C.H.M., D.J. van der Horst and J.P.C.M. van Dongen, Biochemistry 10:4938 (1971).
42. Simon, G., and G. Rouser, Lipids 2:55 (1967).
43. Ackman, R.G., S.N. Hooper and P.J. Ke, Comp. Biochem. Physiol. 39B:579 (1971).
44. de Koning, A.J., J. Sci. Fd. Agric. 17:460 (1966).
45. Zama, K., Mem. Fac. Fish. Hokkaido Univ. 11:1 (1963).
46. Hack, M.H., A.E. Gussin and M.E. Lowe, Comp. Biochem. Physiol. 5:217 (1962).
47. Thiele, O.W., Z. Vergl. Physiol. 42:484 (1959).
48. Hori, T., O. Itasaka, T. Hashimoto and H. Inoue, J. Biochem. (Tokyo) 55:545 (1964).
49. Gray, G.M., and M.G. Macfarlane, Biochem. J. 70:747 (1958).
50. Shieh, H.S., Comp. Biochem. Physiol. 27:533 (1968).

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LETTER TO THE EDITOR

Steroid Acids from EAE Guinea Pig Brain: Identification of 3β-Hydroxy-5-Cholenoic Acid

Sir: We reported the presence of some steroid acids in the brains of guinea pig afflicted with experimental allergic encephalomyelitis (Naqvi, S.H.M., B.L. Herndon, M.T. Kelley, V. Bleisch, R.T. Aexel and H.J. Nicholas, J. Lipid Res. 10:115 [1969]). Lithocholic acid was positively identified, while another unsaturated steroid acid was also reported.

In view of reports on the mass spectra of 5,6-unsaturated 3-hydroxy steroids (Knights, B.A., J. Gas Chromatogr. 5:273 [1967]; Naqvi, S.H.M., Steroids, 22:285 [1973]), we have reexamined the data for the unidentified C-24 steroid acid reported earlier. The mass spectrum of this compound (partly reported earlier) shows major fragments at m/e 213, 215, 225, 231, 233, 255, 277, 303, 311, 315, 339, 351, 355, 370, 373 and 388; some of these are identical to fragments seen in the mass spectrum of a standard sample of methyl 3β-hydroxy-5-cholen-24-oate, while others do not correspond to that standard (Fig. 1). The peaks at 213 and 215 are perhaps due to cleavage of ring D and loss of a molecule of water in two separate compounds, one of which has a double bond. This interpretation is further supported by the presence of peaks at 231 and 233, which would arise by a similar fragmentation with the

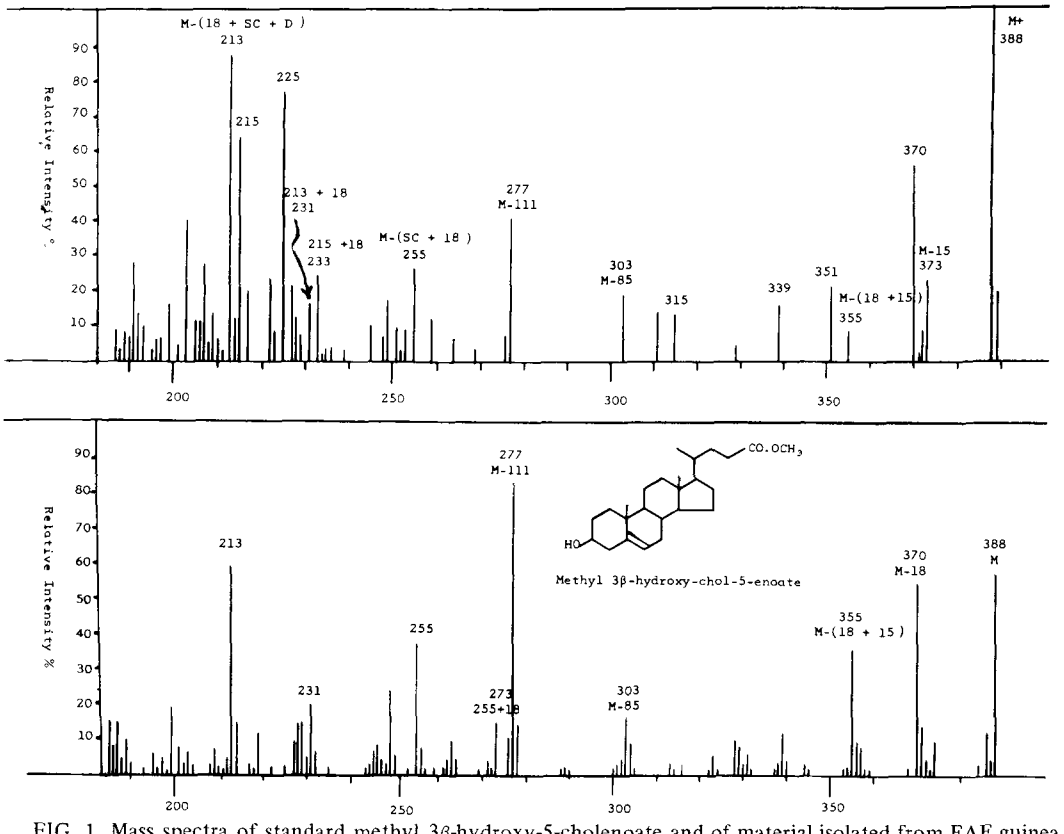

FIG. 1. Mass spectra of standard methyl 3β-hydroxy-5-cholenoate and of material isolated from EAE guinea pig brain.

fragment still retaining the hydroxyl group. The peaks at 255 (M-side chain + 18), 355(M-[18 + 15]), 370 (M-18) and 388 (M) further suggest that at least one of the components is a monohydroxy steroid. Unsaturation is indicated by the fragments at 213, 231 and 255. The relatively large peaks at 277 and 303 correspond to the M-111 and M-85 fragments shown by Knights and by Naqvi to be characteristic of Δ^5-3-hydroxy steroidal compounds. The similarity of the mass spectrum of the unidentified compound with the spectrum of methyl 3β-hydroxy-5-cholen-24-oate confirms that one of the compounds in the mixture is indeed 3β-hydroxy-5-cholen-24-oate.

The fact that the retention time of this material on gas liquid chromatography was longer than the standard methyl 3β-hydroxy-5-cholenoate can be explained by the compound's being in mixture with a substance having longer retention time, thus giving a broad peak.

The material which gave the mass spectrum in Figure 1 resolved into two small peaks when chromatographed as a trimethylsilyl ether; one of the peaks had the same retention time as the standard trimethylsilyl derivative of methyl 3β-hydroxy-5-cholenoate. However the amount of the material was so small that efforts to scan these peaks for mass spectra were not successful.

Since the amounts of steroid acids in EAE brain are very small, it is quite difficult to identify these compounds by the usual methods. It will therefore be interesting to produce EAE in guinea pigs, which already have radioactive-labeled cholesterol in the brain (by injecting labeled mevalonate at birth). In these animals the bile salts produced will also be labeled and therefore may be easier to locate and identify.

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